THIS NUMBER COMPLETES VOLUME 232

VOL. 232 JUNE, 1958 No. 2

THE JOURNAL OF BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

EDITED FOR THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

PUBLISHED MONTHLY
AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE 2, MD.
EDITORIAL OFFICE: YALE UNIVERSITY, NEW HAVEN

FOR THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.

Entered as second-class matter at the Post Office at Baltimore, Md. Acceptance for mailing at special rate of postage provided for in section 1103, Act of October 3, 1917. Authorized on June 20, 1918

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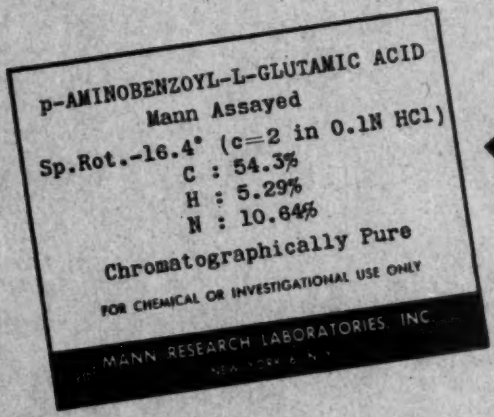

◀ **TYPICAL ANALYSIS**

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HEMOGLOBIN CATABOLISM

II. THE PROTECTION OF HEMOGLOBIN FROM OXIDATIVE BREAKDOWN IN THE INTACT ERYTHROCYTE*

By GORDON C. MILLS and HARVEY P. RANDALL

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(Received for publication, November 11, 1957)

Previous studies have shown that catalase (1, 2) and glutathione peroxidase (3, 4) in hemolysates protect hemoglobin from the destructive effects produced by either hydrogen peroxide or ascorbic acid. Glutathione peroxidase is effective only in the presence of adequate amounts of GSH,[1] which serves as a hydrogen donor. The reaction has been represented by the following equation (4):

$$H_2O_2 + 2GSH \xrightarrow[\text{glutathione peroxidase}]{} 2H_2O + GSSG$$

This protective system has a lasting effect only when the product of the reaction, GSSG, is continuously reduced to GSH. The work of previous investigators has shown that erythrocytes have an effective system for reducing GSSG (5–8). The TPN-linked reactions of the phosphogluconate pathway, in conjunction with glutathione reductase, maintain erythrocyte glutathione in the reduced state.

In the present studies, catalase and the peroxidase-GSH system of intact erythrocytes are shown to protect hemoglobin from oxidative breakdown brought about in the presence of ascorbic acid. When glucose is absent from the incubation medium, the protective effect of the peroxidase-GSH system is lost. This failure is due to the inability of the cell to reduce GSSG. The result is a rapid drop in the GSH level and subsequent loss of the protective effect of the peroxidase-GSH system. The protective effect of catalase in intact erythrocytes is eliminated by azide, a fact which has been demonstrated previously by Foulkes and Lemberg (1).

In addition, studies with hemolysates show that the peroxidase-GSH protective system is effective in the presence of either glucose 6-phosphate, 6-phosphogluconate, or ribose 5-phosphate. These three compounds have been found previously to be effective in reducing GSSG to GSH in erythro-

* This work was supported in part by a research grant from the National Heart Institute of the National Institutes of Health, Public Health Service.

[1] GSH and GSSG denote reduced and oxidized glutathione, respectively; DPNH and DPN, reduced and oxidized diphosphopyridine nucleotide; TPNH and TPN, reduced and oxidized triphosphopyridine nucleotide; ATP, adenosine triphosphate.

cyte hemolysates (9). The maintenance of the GSH concentration at a high level in the hemolysates insures the continued action of the peroxidase-GSH protective system. In these studies, TPN was added to compensate for the partial loss of this essential coenzyme in the preparation of the hemolysate.

Methods

Determination of Methemoglobin and Choleglobin—Methemoglobin, in the presence of choleglobin, was determined from the change in optical density of the solution which accompanies the conversion of cyanmethemoglobin to carboxyhemoglobin (10). The sample was diluted to an appropriate volume and concentration (usually 4 to 6 ml. and 0.05 to 0.15 gm. per cent hemoglobin). Methemoglobin in the sample was converted to cyanmethemoglobin by treatment of the solution with potassium cyanide (3 to 6 mg.). Hemoglobin and oxyhemoglobin in this same solution were then converted to carboxyhemoglobin by treatment of the sample with carbon monoxide. After determination of the optical density of the sample at 570 mμ with a Beckman model DU spectrophotometer, cyanmethemoglobin was converted to carboxyhemoglobin by the addition of sodium dithionite (2 to 3 mg.) and additional carbon monoxide. The optical density of the sample at 570 mμ was again determined (D_{570}), in addition to the optical density at 627 mμ (D_{627}). Since the ratio of the extinction coefficients of carboxyhemoglobin and cyanmethemoglobin at 570 mμ is 1.69:1, the methemoglobin concentration in per cent of total heme compounds may be calculated from the following equations: per cent methemoglobin $= (100(\Delta D_{570})/(0.41\ (x + y)))$; $x = 1.018\ (D_{570} - 0.45\ D_{627})$; and $y = 1.02\ (D_{627} - 0.026\ D_{570})$. The ΔD_{570} is the increase in optical density at 570 mμ produced by treatment of the sample with sodium dithionite and carbon monoxide; x is the absorption at 570 mμ due to carboxyhemoglobin after correction for the absorption of carboxycholeglobin at this wave length; and y is the absorption at 627 mμ due to carboxycholeglobin after correction for the absorption of carboxyhemoglobin at this wave length.

In the determination of methemoglobin by this procedure, it is assumed that the absorption of choleglobin at 570 mμ is not significantly changed by treatment of the sample with sodium dithionite. Experimental verification of the validity of this assumption has been presented previously (10).

The principles of the procedure that was used for the determination of choleglobin in the incubated samples have been described previously (10, 11). The concentration of choleglobin in per cent of total heme compounds was calculated from the following equation: per cent choleglobin $= (100y/(x + y))$. Since the amount of verdohemoglobin in these samples was very small, no corrections were needed to compensate for its presence (11).

Incubation Procedure for Intact Erythrocytes—Erythrocytes from approximately 20 ml. of heparinized rat blood were washed four times with 40 ml. portions of Krebs-Ringer phosphate buffer,[2] pH 7.4. The washed erythrocytes were then diluted to the desired concentration with a Krebs-Ringer phosphate buffer.

All of the solutions that were to be added in these experiments were either prepared as isotonic solutions or were made isotonic by addition of NaCl. Ascorbic acid solutions were neutralized with sodium carbonate before use. When these reagents were added to samples, the amount of 0.15 M NaCl added to that particular sample was decreased accordingly.

50 ml. samples were incubated in 125 ml. Erlenmeyer flasks at 37° with occasional shaking. At the desired time intervals, a 7.5 ml. aliquot was removed for the determination of GSH, and a 0.5 ml. aliquot was removed for determination of choleglobin and methemoglobin. Since large amounts of ascorbic acid interfere in the determination of GSH, most of the added ascorbic acid was removed by centrifugation of the 7.5 ml. aliquot and by discarding the supernatant fluid. The cells were then hemolyzed with 4.0 ml. of H_2O, the sample was treated with carbon monoxide, and the proteins were precipitated with 1 ml. of 25 per cent metaphosphoric acid. The filtrate was frozen and stored until it was analyzed for GSH by the alloxan "305" procedure of Patterson and Lazarow (13).

In order to remove most of the ascorbic acid and any extracellular hemoglobin, the 0.5 ml. aliquot was centrifuged and the supernatant fluid discarded. The cells were hemolyzed by the addition of 0.25 ml. of 0.23 M bicarbonate-carbonate buffer, pH 8.8, and 2.5 ml. of distilled water. The buffer tends to improve the optical clarity of these solutions. Any further reaction of ascorbic acid and hemoglobin was prevented by treating the samples with carbon monoxide immediately after hemolysis. The method used for determination of choleglobin and methemoglobin in the hemolyzed samples has been described above. In order to obtain reproducible spectrophotometer readings, all of the hemolyzed blood samples were centrifuged before the spectrophotometric analysis, even when there was no visible precipitate.

Incubation Procedure for Hemolysates—Erythrocytes from heparinized rat blood were washed once with 3 volumes of a Krebs-Ringer phosphate buffer[2] (pH 7.4) containing nicotinamide (0.011 M). The cells were hemolyzed with distilled water and the isotonicity of the hemolysate was restored by addition of an appropriate amount of a more concentrated solution of Krebs-Ringer buffer (5 times the concentration used above). The

[2] In order to prevent the precipitation of calcium phosphate, the Krebs-Ringer phosphate buffer (12) was modified by reducing the calcium concentration to 2.3×10^{-4} M. In the hemolysate experiments, nicotinamide was added to this buffer at the expense of an osmotically equivalent amount of NaCl.

hemolysates were used without centrifugation. Pyridine nucleotides (Nutritional Biochemicals Corporation) were made up in water as 0.012 M solutions. Solutions of glucose 6-phosphate and ribose 5-phosphate (Schwarz Laboratories, Inc.), 6-phosphogluconate (Sigma Chemical Company), and fructose 1,6-diphosphate (Mann Research Laboratories, Inc.) were prepared by dissolving the barium salts in dilute HCl. Sodium sulfate was added and barium sulfate was removed by centrifugation. The pH of these samples was adjusted to 7 with NaOH before use.

The incubation period was begun by addition of the desired chemicals to the hemolysates. Samples were incubated in test tubes at 37° without being shaken. After various time intervals, aliquots were removed and choleglobin was determined as described above.

Results

Formation of Choleglobin and Methemoglobin in Intact Cells—Foulkes and Lemberg (1) have shown that choleglobin can be produced in intact erythrocytes when they are incubated with ascorbic acid in the presence of azide. In the absence of azide, which inhibits catalase, the above authors could not detect appreciable amounts of choleglobin, even after prolonged periods of incubation. This work suggested that intact cells could be used to study those mechanisms that protect the hemoglobin within the erythrocyte from the destructive effects of ascorbic acid.

In some preliminary experiments, erythrocytes from rat blood were incubated without added glucose for 16 hours. The subsequent addition of ascorbic acid produced considerably more choleglobin within the erythrocyte than was produced by ascorbic acid in cells which were not subjected to the preliminary incubation procedure. Incubation of red cells in the absence of glucose leads to a marked reduction in the intracellular glucose level. The relative ease with which choleglobin formation could be induced in these cells suggested that the protective mechanisms of the cell were closely related to glucose metabolism.

It is difficult to control the extent of depletion of the intracellular glucose when this is brought about by prolonged incubation periods. Consequently, in subsequent experiments the glucose level was reduced by repeated washing of the cells with Krebs-Ringer phosphate buffer. Glucose determinations by the Nelson procedure (14) on filtrates prepared from these washed cells indicated that the intracellular glucose level was below 10 mg. per cent. In Fig. 1 are shown the effects of glucose and azide on choleglobin and methemoglobin formation produced by incubation of these washed cells with ascorbic acid. The samples containing glucose were washed in the same manner as the other cells and the glucose level was restored just before initiation of the experiment. Fig. 2 correlates the

GSH level in the cells in these samples with the formation of choleglobin and methemoglobin.

The greatest choleglobin formation was always achieved in samples which contained azide but no glucose (Sample AA, NaN_3). Even with these cells, there was a lag period before choleglobin formation began, which

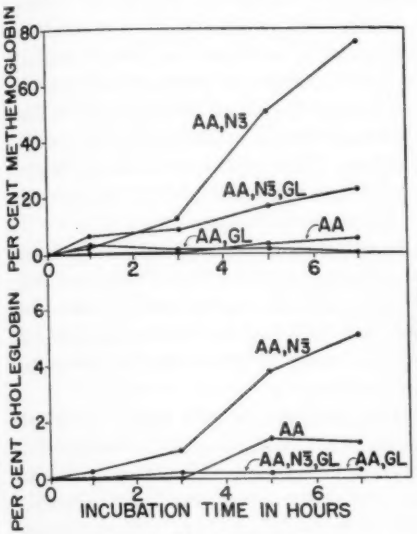

FIG. 1

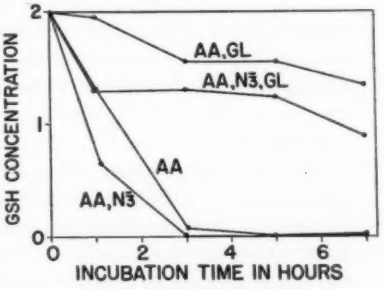

FIG. 2

FIG. 1. The relation of glucose metabolism to choleglobin and methemoglobin formation in washed erythrocytes. The final concentrations of ascorbic acid (AA) and hemoglobin in each of the incubated samples were 1.1×10^{-3} M and 1.0 gm. per cent, respectively. In addition, the components shown adjacent to each curve were present in the following concentrations: sodium azide (NaN_3), 5×10^{-3} M; and glucose (GL), 0.016 M.

FIG. 2. The relation of glucose metabolism to the GSH concentration in washed erythrocytes. The concentrations of the different components were the same as those listed under Fig. 1. The GSH concentration is expressed in terms of mg. per cent GSH in the incubated cell suspension.

corresponded quite closely with the time required for the GSH level in this sample to drop almost to zero. Since catalase in this sample was inactivated by azide, the loss in GSH resulted in the failure of the peroxidase-GSH protective system and subsequent initiation of choleglobin formation.

In the sample containing azide and glucose (Sample AA, NaN_3, GL), there was no significant choleglobin formation despite the fact that the catalase was inhibited by azide. The GSH level of this sample was main-

tained quite well, and consequently the peroxidase-GSH protective system should have been functioning adequately. The absence of choleglobin in this sample, as contrasted to that in the previous sample which contained azide but no glucose, indicates that the peroxidase-GSH protective system was indeed functioning.

In the sample containing ascorbic acid but no glucose (Sample AA), there was a slight choleglobin formation which became significant after about 5 hours of incubation. In this sample the GSH level had dropped almost to zero by 3 hours, and after that time there would be no protective effect due to the GSH-peroxidase system. The small yields of choleglobin in this sample as compared to that in Sample AA, NaN_3, are undoubtedly attributable to the protective effect of catalase. Since catalase seldom provides complete protection (1), it is not surprising that choleglobin was present in Sample AA after the 5th and 7th hours of incubation.

In the sample containing ascorbic acid and glucose (Sample AA, GL), there was no appreciable choleglobin formation, and the GSH level was well maintained throughout the course of the experiment. Thus, the results with this sample are in accord with the view that both catalase and the peroxidase-GSH system were functioning in protecting erythrocyte hemoglobin.

The presence of azide in two of these samples brought about a marked production of methemoglobin (Fig. 1, AA, NaN_3 and AA, NaN_3, GL). It appears that this effect of azide is due to trapping of methemoglobin as methemoglobin azide or to inhibition of the enzyme systems responsible for the reduction of methemoglobin to hemoglobin (15). There was no significant formation of methemoglobin in either of the other two samples (Fig. 1, AA, and AA, GL). Since, in the coupled oxidation of hemoglobin and ascorbic acid there is always more methemoglobin produced than choleglobin, it appears that in these samples any methemoglobin is being reduced as rapidly as it is formed. Methemoglobin reduction is also dependent upon glucose metabolism; the results with Sample AA indicate, therefore, that the substrates and coenzymes responsible for methemoglobin reduction are not depleted as rapidly as those required for the reduction of GSSG. Gibson (15) has shown that lactic acid will effectively serve as a hydrogen donor in the reduction of methemoglobin. Presumably, there is sufficient lactic acid present in this sample to prevent the accumulation of any methemoglobin. It is evident from the results with Sample AA, NaN_3, GL, as well as from studies with crystalline hemoglobin, that the peroxidase-GSH system is not nearly as effective in preventing methemoglobin formation as it is in protecting hemoglobin from oxidative breakdown.

The results shown in Fig. 1 have been qualitatively confirmed in other

experiments carried out in essentially the same manner. Methemoglobin formation was always highest in samples containing azide but no glucose, and a significant amount of methemoglobin also was formed in the presence of azide and glucose. Choleglobin formation was most marked in the samples containing azide but no glucose. In the absence of glucose, choleglobin formation has always been significantly greater than in the presence of glucose.

Protection of Hemoglobin from Oxidative Breakdown in Erythrocyte Hemolysates—In order to relate more specifically the effect of glucose upon the protective mechanisms of red cells, experiments were carried out with the use of hemolysates. The two primary metabolic defects of hemolysates are their inability to phosphorylate glucose, owing to a loss of ATP, and breakdown of the normal hydrogen transfer mechanisms, owing to a loss of DPN and TPN (16). Consequently, in these studies, hemolysates were fortified with the desired coenzyme, with the use of either glucose 6-phosphate, 6-phosphogluconate, ribose 5-phosphate, or fructose 1,6-diphosphate as substrates. Since preliminary data suggest that glutathione may be partially destroyed in hemolysates, GSSG also was added to provide an adequate source of GSH.

The results of these experiments are shown in Fig. 3. When only ascorbic acid was added to the hemolysate, hemoglobin breakdown began after approximately 90 minutes of incubation (Curve AA). The addition of GSSG to the hemolysate protected hemoglobin from oxidative breakdown for about 180 minutes (Curve AA + GSSG). Apparently there were enough glucose 6-phosphate and TPN present in the hemolysate to reduce some of the GSSG to GSH, thus maintaining the protective effect of the peroxidase-GSH system for a longer period. The results with the samples which contained either glucose 6-phosphate, 6-phosphogluconate, or ribose 5-phosphate were in marked contrast to those described above. Here, hemoglobin was protected from oxidative breakdown for the full 360 minutes. The protection of hemoglobin in these samples lasted as long as the protection provided by GSH alone (Curve AA + GSH). The effect of these three compounds in hemolysates is in accord with previous studies which show that GSSG is converted to GSH by TPNH in the presence of glutathione reductase. The TPNH is produced by dehydrogenation of glucose 6-phosphate or of 6-phosphogluconate. Ribose 5-phosphate is probably utilized after its conversion to glucose 6-phosphate. Fructose 1,6-diphosphate and DPN were totally ineffective in protecting hemoglobin from oxidative breakdown. Since DPN-linked systems are also reported to reduce GSSG slowly (8), the interpretation of these results with DPN and fructose 1,6-diphosphate must await further experimentation.

The results of these experiments with hemolysates are in accord with the

results of the experiments utilizing intact cells. In both cases, the peroxidase-GSH system is effective in protecting hemoglobin from oxidative breakdown as long as an adequate amount of GSH is present. In intact cells an adequate GSH level is maintained as long as glucose is present in the incubation medium. In hemolysates, substrates which will serve as hydrogen donors to TPN are effective in the maintenance of GSH at an adequate level.

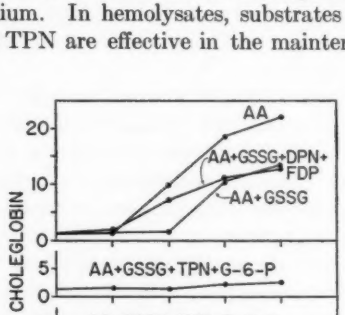

FIG. 3. The relation of red cell metabolism to the protection of hemoglobin from oxidative breakdown in hemolysates. The final concentrations of ascorbic acid (AA) and hemoglobin in each of the incubated samples were 3.5×10^{-3} M and 1.0 gm. per cent, respectively. In addition, the components shown adjacent to each curve were present in the following concentrations: GSH and GSSG, 8.0×10^{-4} M; TPN and DPN, 4.0×10^{-4} M; glucose 6-phosphate (G-6-P), ribose 5-phosphate (R-5-P), 6-phosphogluconate (6-PG), and fructose 1,6-diphosphate (FDP), 5.6×10^{-3} M. Each sample was made to a final volume of 6.0 ml. by the addition of the required amount of 0.15 M NaCl.

There was no significant amount of methemoglobin produced in any of the hemolysate samples during the incubation period. Apparently, all of the samples contained sufficient lactic acid and the cofactors needed to prevent the accumulation of methemoglobin.

DISCUSSION

The coupled oxidation of ascorbic acid and oxyhemoglobin probably leads to the intermediate formation of H_2O_2 or a hemoglobin-H_2O_2 complex (17). Since ascorbic acid acts as a hydrogen or electron donor in the above reaction, other hydrogen donors which bring about the oxidative breakdown of hemoglobin probably react in an analogous manner. Apparently such compounds as epinephrine (18), phenylhydrazine (19), and acetyl-

phenylhydrazine (9) react with oxyhemoglobin to yield H_2O_2 or a complex, since the peroxidase-GSH system protects hemoglobin from oxidative breakdown in the presence of these compounds.[3] Certain drugs which have hemolytic effects in drug-sensitive individuals appear to react similarly *in vivo* to phenylhydrazine (20). These drugs produce a marked reduction of GSH in erythrocytes of drug-sensitive individuals (21), owing to a deficiency of glucose-6-phosphate dehydrogenase within the erythrocyte (5). Because of this deficiency, the ability of these red cells to reduce GSSG to GSH is markedly lowered. The following sequence of reactions provides a logical explanation for the low GSH level in drug-sensitive individuals. The drug, or a metabolite of the drug, reacts with oxyhemoglobin to produce hydrogen peroxide. The GSH peroxidase catalyzes the destruction of H_2O_2, and GSH is oxidized to GSSG. In normal red cells, GSSG is reduced as rapidly as it is formed, but in drug-sensitive cells the oxidation of GSH proceeds at a rate which exceeds the capacity of the cell to reduce GSSG. Here, the erythrocyte GSH level drops and a marked hemolysis follows.

The necessity of including oxyhemoglobin in the sequence of reactions was shown by Beutler, Robson, and Buttenweiser (9) in their studies with acetylphenylhydrazine. With hemolysates, the acetylphenylhydrazine brought about a marked increase in the rate of oxidation of GSH when oxyhemoglobin was present, even though the oxyhemoglobin was not significantly altered in the process. Since acetylphenylhydrazine brings about the oxidative breakdown of hemoglobin in solutions of crystalline hemoglobin,[3] the protection of hemoglobin from oxidative breakdown in the experiments of Beutler *et al.* (9) must have been due to the presence in the hemolysates of the peroxidase-GSH system. In the process of protecting the hemoglobin from oxidative breakdown, the GSH is oxidized to GSSG. The relation between the lowered GSH level and the hemolysis of the erythrocytes is not yet clear. Previous workers have shown that chemicals which oxidize or react with erythrocyte sulfhydryl compounds produce an increased rate of hemolysis (22, 23).

The authors wish to acknowledge the technical assistance of Miss Dolores Casimere and Miss Freddie Craven in the studies with hemolysates.

SUMMARY

Studies have been conducted with intact erythrocytes to show the relationship of cell metabolism to the ability of the cell to protect hemoglobin from oxidation. Reduction of the glucose level of the cells resulted in a marked decrease in the efficiency of the protective mechanisms of the cell. Evidence is presented which indicates that the over-all effect of the glucose

[3] Mills, G. C., unpublished results.

is to maintain glutathione in the reduced state. In the presence of an adequate amount of reduced glutathione (GSH), the peroxidase protects hemoglobin from oxidative breakdown by catalyzing the destruction of hydrogen peroxide. Azide does not interfere with this peroxidase-GSH protective system but it does eliminate the additional protective effect of catalase.

Experiments with erythrocyte hemolysates also showed the relationship of cell metabolism to the protection of hemoglobin. Either glucose 6-phosphate, 6-phosphogluconate, or ribose 5-phosphate in the presence of added oxidized triphosphopyridine nucleotide and oxidized glutathione markedly increased the ability of hemolysates to protect hemoglobin from oxidative breakdown.

The relationship of these protective systems to the hemolytic effects of certain drugs is also discussed.

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BIOGENESIS OF YEAST STEROLS

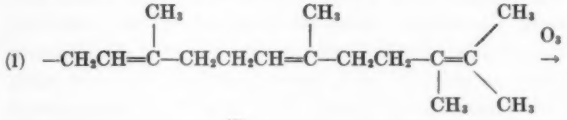
III. THE ORIGIN OF CARBON 28 OF ERGOSTEROL*

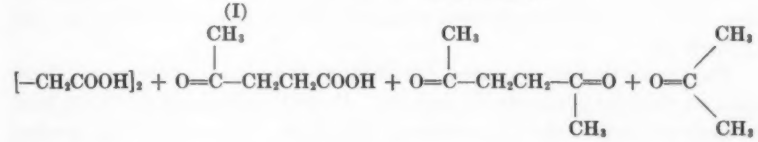
By GEORGE J. ALEXANDER,† ALLEN M. GOLD, AND ERWIN SCHWENK

(From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts)

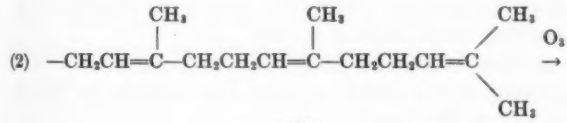
(Received for publication, December 2, 1957)

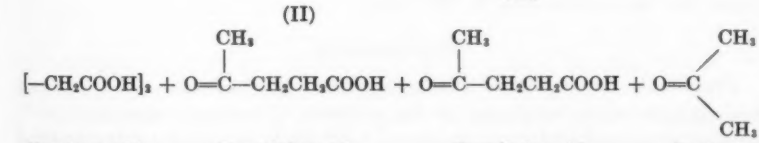
The generally accepted scheme of sterol biogenesis does not account for the presence of the methyl group attached to C-24 of ergosterol. It has been shown that this methyl group is not derived from acetic acid (1). In 1955 Robinson presented the hypothesis that condensation of 1 mole of propionate with 17 moles of acetate would explain the existence of this "extra" carbon atom (2). A similar scheme was proposed by Woodward





Succinic acid Levulinic acid 2,5-Hexanedione Acetone





Succinic acid Levulinic acid Levulinic acid Acetone

* This work was supported by the United States Public Health Service grant No. C321, an Institutional Grant from the American Cancer Society, The Schering Corporation, Bloomfield, New Jersey, and the Jane Coffin Childs Memorial Fund.

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for the biosynthesis of certain antibiotics and branched chain fatty acids such as tuberculostearic acid (3). Robinson's suggestion implied that a methyl squalene (I), rather than squalene (II), is a precursor of ergosterol. This methyl squalene, on ozonization, would yield 1 mole of 2,5-hexanedione instead of 1 of the 4 moles of levulinic acid given by squalene (4). If radioactive propionate were incorporated into the hypothetical methyl squalene, it would be found in the hexanedione.

An alternative explanation for the origin of the "extra" methyl group of ergosterol predicates identical precursors for cholesterol and ergosterol beyond squalene, and the introduction of a 1-carbon unit at a later stage of the biosynthesis. Both hypotheses were investigated and the results of these investigations are given below.

General Procedure

In whole yeast experiments, dry bakers' yeast (Fleischmann's) was suspended in Tris[1]-HCl buffer with yeast hydrolysate (Nutritional Biochemicals Corporation, Cleveland, Ohio) as a source of the cofactors. After addition of radioactive substrate, the suspension was divided into aliquots and incubated aerobically on a rotary shaking table at room temperature. Yeast homogenate was prepared by stirring a suspension of dry yeast in aqueous glycerol solution, followed by centrifugation and dialysis (5).

The incubation was stopped by addition of an equal volume of 20 per cent methanolic potassium hydroxide. Pooled contents of the flasks were hydrolyzed, acidified, and extracted with ether. The extract was washed thoroughly with alkali to remove acidic material. The non-saponifiable residue was treated with digitonin and the sterols were isolated from the digitonides as described before (6). The fatty acids were recovered by acidification and extraction of the alkaline washes.

Unless otherwise specified, all labeled substances contained 1 mc. of C^{14} per mmole. Radioactivity was assayed in a gas flow counter, in which 1 mc. was equivalent to 3×10^8 c.p.m.

EXPERIMENTAL

Propionate As Ergosterol Precursor—Resting yeast and cell-free yeast homogenates were incubated in the presence of sodium propionate-1-C^{14}, sodium propionate-2-C^{14}, or propionyl-1-C^{14}-CoA, prepared by the method of Simon and Shemin (7), with sodium acetate-1-C^{14} as a standard for comparison. All propionate experiments were uniformly negative. No

[1] The following abbreviations have been used: adenosine triphosphate, ATP; diphosphopyridine nucleotide, DPN; coenzyme A, CoA; tris(hydroxymethyl)-aminomethane, Tris.

differences were noted between the incorporation of propionate-1 or 2-C^{14} or the CoA derivative. A typical series of experiments is shown in Table I.

Degradation of Yeast Squalene—About 25 gm. of yeast non-saponifiable matter obtained from the mother liquors of ergosterol manufacture[2] were chromatographed on an alumina column. The pentane eluate yielded 1.63 gm. of squalene-containing oil. This substance was dissolved in 75 ml. of ethyl acetate and cooled to −70° in a dry ice bath. A white precipitate similar to material encountered before (8) was removed by filtration and the residue from the filtrate was dissolved in pentane and treated with active charcoal. After filtration through Celite, the solvent was evapo-

TABLE I

Incorporation of Propionic Acid into Sterols

System	Substrate	Amount added	C^{14} recovered in sterols
		μc.	*c.p.m. per mg.*
Whole yeast	Sodium acetate-1-C^{14}	16	1650
" "	" propionate-1-C^{14}	16	0.7
" "	" propionate-2-C^{14}	16	0.5
" "	Propionyl-2-C^{14}-CoA	16	0.6
Homogenate	Sodium acetate-1-C^{14}	10	4200
"	" propionate-1-C^{14}	10	2
"	" propionate-2-C^{14}	10	2
"	Propionyl-2-C^{14}-CoA	10	3

Whole yeast: Each batch consisted of four flasks containing 1.5 gm. of dry bakers' yeast, 10 ml. of 0.1 M Tris-HCl buffer (pH 7), 0.1 gm. of yeast hydrolysate (Nutritional Biochemicals Corporation), and 4 μc. of labeled substrate. Incubated at room temperature for 48 hours. Homogenate: Each batch consisted of four flasks containing 4 ml. of homogenate, equivalent to 1 gm. of dry bakers' yeast, 0.0004 M ATP, DPN, and CoA, 0.1 M Tris-HCl buffer, and 2.5 μc. of labeled substrate. Incubated at room temperature for 48 hours.

rated, leaving 0.96 gm. of viscous oil, which was then carefully rechromatographed on 100 gm. of Alcoa alumina. The pentane fractions containing squalene were combined, yielding 0.38 gm. of pure colorless product. The infrared spectrum of this product was identical to the spectrum of the best authentic sample of squalene available.

The purified squalene (0.33 gm.) was dissolved in ethyl acetate and treated with excess ozone at −70°. The solvent was removed by gentle warming *in vacuo* and the residue treated overnight with H_2O_2 in glacial acetic acid. Water was then added and the solution neutralized with

[2] The authors thank Dr. F. Reiff, Director of the Research Laboratories of Zellstoff-Fabrik Waldhof, Mannheim-Waldhof, Germany, for the generous gift of this material.

K_2CO_3 and partially distilled. Yellow crystals precipitated when the distillate was treated with 2,4-dinitrophenylhydrazone reagent solution (9). The isolated crystals dissolved when treated with 5 ml. of boiling methanol. On cooling, the methanol deposited 53 mg. of yellow crystals, m.p. 125.0-125.6° (authentic acetone-2,4-dinitrophenylhydrazone melted at 126.0-127.0°). Control experiments indicated that the presence of only a few mg. of 2,5-hexanedione would be detectable by the insolubility of its 2,4-dinitrophenylhydrazone in methanol. Crude levulinic acid was isolated, as the 2,4-dinitrophenylhydrazone, in 62 per cent yield from the undistilled residue.

A confirmatory experiment was carried out with 5 mg. of radioactive yeast squalene, specific activity 5000 c.p.m., prepared by incubation of yeast homogenate with carboxyl-labeled acetate. After ozonization, 10 mg. of 2,5-hexanedione were added to the reaction mixture, which was then treated with H_2O_2 as before. The distillate gave a heavy precipitate with 2,4-dinitrophenylhydrazine. The product was washed with boiling methanol and recrystallized from pyridine, yielding 10 mg. of pure 2,5-hexanedione-bis-2,4-dinitrophenylhydrazone, which proved devoid of radioactivity. Levulinic acid (15 mg.) was added to the non-volatile residue from the distillation and reisolated as the 2,4-dinitrophenylhydrazone, m.p. 204°, and specific activity 200 c.p.m. If squalene had been the only radioactive component of the starting material, the levulinic acid 2,4-dinitrophenylhydrazone should have given a specific activity of 275 c.p.m.

Comparison of Various 1-Carbon Donors As Precursors of Ergosterol—Possible sources of the "extra" group of ergosterol were investigated in parallel experiments in which aliquots of one yeast homogenate preparation incubated with various radioactive substrates were used. The non-saponifiable fraction, isolated as usual, was diluted with 10 mg. of ergosterol, precipitated with digitonin, and the recovered ergosterol was recrystallized to constant specific activity. The fatty acids of the saponifiable fraction were counted directly. Two typical experiments involving sodium acetate-1-C^{14}, sodium propionate-2-C^{14}, sodium bicarbonate-C^{14}, formaldehyde-C^{14}, sodium formate-C^{14}, choline-*N*-methyl-C^{14}, serine-3-C^{14}, and methionine-methyl-C^{14} are shown in Table II.

The optimal duration of incubation of yeast homogenates with methionine-methyl-C^{14} was determined by incubating aliquots of homogenate for increasing periods of time. The capacity of the system to utilize methionine was established by incubating aliquots of homogenate with a constant quantity of methionine-methyl-C^{14} and with increasing amounts of non-radioactive methionine as a diluent. The results of these two experiments are shown in Figs. 1 and 2.

If methionine-methyl-C^{14} were the specific precursor of the "extra" methyl group of ergosterol, then squalene, lanosterol, and zymosterol should prove inactive since they do not possess that methyl group. There-

TABLE II

Comparison of Different C^{14} Donors As Precursors of Ergosterol and Fatty Acids

Experiment No.	Source of C^{14}	Microcurie	Fatty acids		Non-saponifiable fraction		Ergosterol	
			Total counts	Per cent yield	Total counts	Per cent yield	Total counts	Per cent yield
1a	Sodium acetate-1-C^{14}	1.0	940	0.30	3,670	1.20	1,400	0.47
b	" propionate-2-C^{14}	1.0	98	0.05	1,230	0.43	250	0.08
c	Formaldehyde-C^{14}	1.0	36	0.01	1,330	0.43	515	0.17
d	$NaHC^{14}O_3$	1.0	150	0.05	2,300	0.75	760	0.25
e	L-Methionine-methyl-C^{14}	1.0	640	0.21	60,600	20.2	42,600	14.2
2a	Choline-*N*-methyl-C^{14}	0.5	270	0.18	700	0.47	510	0.34
b	Sodium formate-C^{14}	0.5	430	0.29	8,640	5.8	2,700	1.87
c	DL-Serine-3-C^{14}	0.5	470	0.32	16,600	11.0	8,150	5.43
d	L-Methionine-methyl-C^{14}	0.5	300	0.20	37,200	24.8	18,750	12.5

The homogenate is the same as in the experiment described in Table I. Incubated at room temperature for 48 hours. The ergosterol yields are computed from the experimental specific activities and the total weights of carrier added.

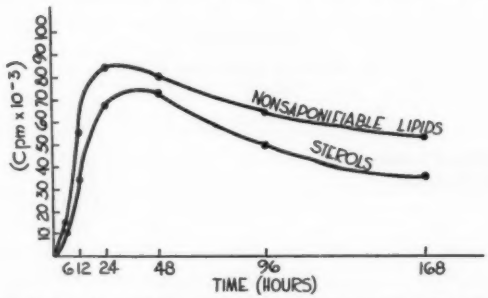

FIG. 1. Optimal duration of incubation with methionine. The same homogenate as in the experiment described in Table I. 1 μc. of methionine-methyl-C^{14} was added to each flask.

fore an experiment was designed to determine whether these substances contain significant amounts of radioactivity. Three aliquots of yeast homogenate were incubated with methionine-methyl-C^{14} for 48 hours at room temperature. The non-saponifiable fraction was isolated and diluted with 100 mg. of ergosterol, yielding a total of 106 mg. of specific activity 2125 c.p.m. Three crystallizations from very dilute methanol solutions,

with great losses at each step, gave pure sterol, specific activity 1555 c.p.m., λ_{max} 271, 282, 293 mμ (ϵ_{max} 11,950, 12,700, and 7150, respectively). The residue from the combined mother liquors was acetylated with acetic anhydride in pyridine and treated with excess maleic anhydride in xylene in order to remove the remaining ergosterol (6). Squalene was separated

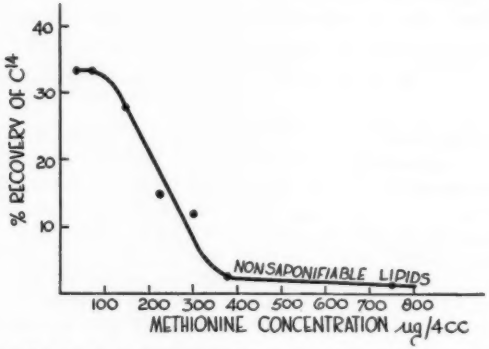

FIG. 2. Capacity of yeast homogenate to convert methionine to non-saponifiable lipides. The same homogenate as before. 0.5 μc. (0.075 mg.) of methionine-methyl-C^{14} in each flask plus additional unlabeled methionine, as indicated. 48 hour incubation.

TABLE III

Isolation of Ergosterol-28-C^{14}

	C.p.m.	Per cent yield
Methionine-methyl-C^{14} used	450,000	
Non-saponifiable fraction	225,000	50
Ergosterol	161,700	36
Squalene	20	0.004
Lanosterol	100	0.02
Zymosterol	100	0.02

Three flasks of the usual homogenate, with 0.5 μc. of methionine-methyl-C^{14} in each. Incubated for 48 hours.

from the residue by chromatography on alumina. Zymosterol and lanosterol in the sterol fraction of the chromatogram were separated by differential precipitation with digitonin (10). Carrier lanosterol and zymosterol were added to the appropriate fractions and the sterols purified by paper chromatography (11). No radioactivity persisted with the lanosterol or zymosterol fractions, even though the accuracy of the procedure permitted detection of as little as 100 c.p.m. over background (Table III).

Degradation of Ergosterol-C^{14}—A stepwise degradation of ergosterol derived from methionine-methyl-C^{14} was carried out in order to prove that the radioactivity is concentrated in C-28. Small amounts of ergosterol-C^{14} synthesized in yeast homogenates from acetate-1-C^{14} and from methionine-methyl-C^{14} were available and were used in a preliminary experiment. A total of 25 mg. of "acetate" ergosterol, specific activity 388 c.p.m., and 30 mg. of "methionine" ergosterol, specific activity 269 c.p.m., were ozonized according to the procedure of Hanahan and Wakil (1). In each case, the non-volatile residue from the steam distillation was extracted and assayed for radioactivity. The steam-volatile material, α,β-dimethylbutyraldehyde from the side chain of ergosterol, was converted to the 2,4-dinitrophenylhydrazone and purified by chromatography. The results indicated

TABLE IV

Degradation of Ergosterol Acetate Derived from Methionine-methyl-C^{14}

Substance	C.p.m. per mmole carbon	
	Experimental	Theoretical
Ergosterol acetate	340	
α,β-Dimethylbutyrate	1730	1,700
C-23 (as $BaCO_3$)	0	0
Iodoform (C-28)	7030	10,200

Ergosterol prepared by incubation of 25 gm. of dry yeast in 480 ml. of distilled water, 0.17 M glucose, 0.015 M NaAc, 0.001 M NaCl, $MgSO_4$, 0.0001 M nicotinamide, 0.00001 M ATP, 0.00001 M DPN, 0.1 M Tris-HCl buffer (pH 7), and 30 μc. (4.5 mg.) of L-methionine-methyl-C^{14}. 24 hour incubation. For details of purification of the sterol, see the text. All samples were assayed as $BaCO_3$.

that the radioactivity from acetate-1-C^{14} was distributed throughout the molecule, while the radioactivity from methionine-methyl-C^{14} was found mainly in the side chain of ergosterol.

The experiment was repeated with a larger quantity of "methionine" ergosterol carefully chromatographed over 45 gm. of alumina (Woelm, grade I). A small portion of the ergosterol was recrystallized from acetone, ethanol, and methanol until, after eight crystallizations, a constant specific activity of 770 c.p.m. was reached. A sample of this purified ergosterol was chromatographed on Whatman No. 1 paper in the kerosene-alcohol system for 48 hours. A single radioactive spot, coincident with the location of ergosterol, was observed, demonstrating the radiopurity of the substance.

The remaining crude ergosterol was acetylated and decolorized with Norit in pentane. The white product was recrystallized four times from ethanol to constant specific activity, 700 c.p.m., which corresponds to a specific activity of 770 c.p.m. for the free sterol. A total of 52.8 mg. of

this substance was mixed with 950 mg. of non-radioactive ergosterol acetate, yielding 1.0 gm. of substance, specific activity 36 c.p.m.

This ergosterol acetate was ozonized as before. The 6-carbon fragment from the side chain was isolated as sodium α,β-dimethylbutyrate, C-23 as $BaCO_3$, and C-28 as iodoform. All samples were converted to $BaCO_3$, the sterol and the sodium α,β-dimethylbutyrate by dry combustion, and the iodoform by the Van Slyke oxidation (Table IV).

DISCUSSION

It is apparent that neither the radioactive sodium propionate nor its CoA derivative was incorporated into ergosterol. The small quantities of radioactivity found in ergosterol in these experiments can easily be accounted for by partial degradation of the propionate-C^{14} and reincorporation of its components via the metabolic mill.

Since ergosterol is the main sterol of yeast, constituting about 90 per cent of the sterol content, any precursor of ergosterol could be expected to be present in sizable quantities, as compared to corresponding precursors of secondary yeast sterols such as zymosterol. If propionate were to enter the biosynthetic sequence together with acetate, the major portion of the "squalene" fraction from yeast should exist as "methyl squalene." Ozonization of yeast squalene gave acetone and levulinic acid but no 2,5-hexanedione, which would be formed from a methyl squalene. Degradation of yeast squalene synthesized from acetate-1-C^{14}, with use of carrier levulinic acid and 2,5-hexanedione, yielded radioactive levulinic acid but inactive diketone, which proves that squalene was degraded as expected and that no methyl squalene was present. Even 10 per cent of such admixture would have been sufficient to permit detection through the radioactivity of the 2,5-hexanedione. These findings prove that propionic acid is not a precursor of C-28 in the ergosterol molecule.

As can be seen from Table II, methionine, serine, and formate[3] were better sources of C^{14} than the other compounds tested, which contributed only negligibly to the radioactivity of ergosterol. The yields from methionine were excellent, exceeding those previously obtained with any radioactive precursor of ergosterol in yeast, including acetate. In later incubations yields as high as 68 per cent in the non-saponifiable fraction and 51 per cent in the sterols were obtained (14).

Fig. 1 demonstrates that incorporation of C^{14} from methionine into the non-saponifiable fraction reaches a maximum after 24 hours, while incor-

[3] While the work reported in this and the following paper was in progress, Danielson and Bloch (12) reported on the incorporation of formate into C-28 in ergosterol and Dauben *et al.* (13) described the conversion of formate into the corresponding carbon atom in eburicoic acid.

poration into sterols is highest after 48 hours of incubation. At the latter time 90 per cent of the radioactivity present in the non-saponifiable material resides in sterols. It is interesting to note that the total radioactivity of the sterols decreases after this time. Autoxidation, which would prevent precipitation with digitonin, may account for some of this decrease. A conversion of ergosterol to compounds analogous to the bile acids, similar to the conversion of cholesterol in mammalian tissues, would also cause a loss of radioactivity, since it involves a degradation of the side chain in which all the C^{14} is concentrated.

The capacity of the homogenate to convert methionine to non-saponifiable lipides, as shown in Fig. 2, indicates that a maximum of 1 μmole of methionine can be efficiently utilized and consequently a maximum of 1 μmole of ergosterol can be synthesized in a standard incubation. However, only 30 per cent of the radioactivity is utilized in ergosterol synthesis, and therefore only about 140 γ of ergosterol are synthesized in the 4 ml. of homogenate, in a 48 hour incubation. Since the homogenate contains 1.5 mg. of proteins, the rate of ergosterol synthesis is approximately 0.2 γ per mg. of protein per hour.

The analysis of non-saponifiable lipides from an incubation with methionine-methyl-C^{14} (Table III) shows that 72 per cent of the radioactivity resides in ergosterol, and none in zymosterol and lanosterol. In a comparable experiment (10) with the same type of yeast homogenate incubated with acetate-1-C^{14}, 36 per cent of the radioactivity resided in ergosterol, 4.5 per cent in zymosterol, and 2.7 per cent in lanosterol. This proves that all three compounds are derived from acetate, while only ergosterol is derived from methionine.

The data from the stepwise degradation of the side chain of ergosterol confirm this result in demonstrating that the incorporation of C^{14} is limited to C-28 when methionine-methyl-C^{14} is used as a source of radioactivity. The sodium α,β-dimethylbutyrate contains all the radioactivity of the parent sterol, and C-23 is completely devoid of activity, suggesting that no randomization of C^{14} occurred.[4] The low activity of the $BaCO_3$ derived from C-28 must be ascribed to the technique of wet combustion of iodoform. Similar low results have been obtained by other investigators (16).

It was shown in a previous paper from this laboratory that squalene and lanosterol of animal origin which lack the "extra" methyl group are converted by yeast homogenates into ergosterol (10). These experiments agree with the present findings that propionic acid is not a precursor of ergosterol. These observations and the data on methionine suggest that

[4] The results of a degradation of ergosterol reported in a preliminary communication from this laboratory (15) were obtained with another less pure sample of the sterol.

"extra" methyl groups in natural substances, such as certain mold metabolites, branched chain fatty acids, and terpenes are not derived from propionic acid as proposed by Robinson (2) and Woodward (3) but from the methyl group in methionine. Birch *et al.* have recently reached a similar conclusion concerning the origin of methyl groups in a variety of natural products, based on their study of the biosynthesis of mycophenolic acid (17).

The technical assistance of Miss Nancy Henry is gratefully acknowledged.

SUMMARY

1. Propionic acid, examined as a potential precursor of the side chain of ergosterol, was not incorporated into the sterol. Squalene from yeast, degraded by ozonolysis, was found to be identical with squalene from animal sources.

2. Methionine-methyl-C^{14}, serine-3-C^{14}, and sodium formate-C^{14} were incorporated into ergosterol in yeast homogenates, methionine being the most efficient source of radioactivity for the sterol.

3. Choline-*N*-methyl-C^{14}, formaldehyde-C^{14}, and sodium bicarbonate-C^{14} were incorporated only to a negligible extent.

4. The maximal incorporation of methionine-methyl-C^{14} occurred in 48 hours of incubation. The maximal rate of synthesis was found to be 0.2 γ of sterol per mg. of protein per hour.

5. With methionine-methyl-C^{14} as the source of radioactivity, only ergosterol was found to be radioactive; zymosterol and lanosterol were devoid of radioactivity. A stepwise degradation of ergosterol acetate showed that all the radioactivity resided in C-28.

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BIOGENESIS OF YEAST STEROLS

IV. TRANSMETHYLATION IN ERGOSTEROL SYNTHESIS*

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(Received for publication, December 2, 1957)

Methionine was found to be more efficient than formate, serine, bicarbonate, formaldehyde, or choline as a source of the C-28 methyl group of ergosterol in yeast (1). While transfer of an intact methyl group to carbon has not been previously recorded, oxidation of the methyl group of methionine to formate has been demonstrated and formate-C^{14} has been shown to give rise to C-28 of ergosterol (2). Nevertheless, preliminary experiments in this laboratory indicated that a true transmethylation may occur (3). The experiments described in this paper were designed to elucidate the mechanism of the transfer of the 1-carbon unit from methionine to sterol.

General Procedures

All experiments were carried out with cell-free yeast homogenate prepared, as before, from dry bakers' yeast by stirring in glycerol suspension (4). Each incubation consisted of homogenate corresponding to 1 gm. of yeast and containing 1.5 mg. of protein with a radioactive substrate and such additional substances as indicated in Tables I to IV. Incubations were carried out at room temperature on a rotary shaking machine. The non-saponifiable lipides and sterols were isolated as before (1). 10 mg. of carrier ergosterol were added to the non-saponifiable fraction before the isolation of sterols. Radioactivity was determined in a gas flow counter in which 1 mc. equals 3×10^8 c.p.m.

EXPERIMENTAL[1]

Utilization of Methionine, Sodium Formate, and Serine—The relationship between methionine and formate as precursors of ergosterol was studied in a series of competitive inhibition experiments in which excess of one of the compounds was used to inhibit the incorporation of the other. Homo-

* This work was supported by the United States Public Health Service grant No. C321, an Institutional Grant from the American Cancer Society, and the Jane Coffin Childs Memorial Fund.

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[1] The following abbreviations have been used: tris(hydroxymethyl)aminomethane, Tris; adenosine triphosphate, ATP.

cysteine and folic acid, which catalyzes the condensation of formate and homocysteine to methionine, were used to ascertain the relative importance of *de novo* methyl synthesis. Aminopterin, a folic acid antagonist (5), provided complementary evidence. The results of a number of typical

TABLE I

Methionine and Formate As Precursors of Ergosterol

Experiment No.	Source of C^{14}	Additions	Recovery			
			Non-saponifiable		Sterols	
			Total activity $\times 10^{-3}$	Per cent	Total activity $\times 10^{-3}$	Per cent
1a	Formate		8.6	5.8	6.6	4.4
b	"	DL-Homocysteine	15.2	10.0	12.0	8.0
c	"	" + folic acid	25.8	17.0	21.8	14.5
d	"	" + Aminopterin	3.5	2.3	1.5	1.0
e	"	L-Methionine	0.5	0.3	0.3	0.2
f	L-Methionine		37.2	25.0	29.2	19.5
g	"	Formate	31.5	21.0	27.5	18.3
h	"	" + DL-homocysteine	8.6	5.7	7.4	4.9
i	"	" + " + folic acid	2.0	1.3	1.9	1.3
j	"	Formate + DL-homocysteine + Aminopterin	26.0	17.3	24.0	16.0
2a	Formate				3.0	2.0
b	"	L-Methionine			1.2	0.8
c	L-Methionine				29.4	19.9
d	"	Formate + DL-homocysteine + folic acid			1.9	1.3
e	"	Formate + DL-homocysteine + Aminopterin			24.4	16.3

Each flask contained 4 ml. of yeast homogenate made from 1 gm. of dry yeast, 0.1 M Tris-HCl buffer (pH 6), 0.0002 M ATP, and 0.5 μc. of C^{14} (1 mc. per mmole). Additions, where indicated, were homocysteine, 5 mg.; folic acid, 1 mg.; Aminopterin, 2 mg.; methionine, 15 mg.; sodium formate, 5 mg. Incubated at room temperature for 48 hours.

experiments are shown in Tables I and II. Addition of ethionine, the *S*-ethyl analogue of methionine, cut the yield from methionine in half. A hydroxy analogue of methionine, α-hydroxy,γ-methylmercaptobutyric acid proved even more inhibitory.

Since serine-3-C^{14} was found to be a good 1-carbon donor (1), non-radioactive serine was added to the homogenate along with methionine-methyl-C^{14}. Surprisingly, a considerable enhancement of the incorporation of

methionine-methyl-C^{14} was observed (Table III). This effect was consistently noted in many experiments.

TABLE II

Influence of Folic Acid

Experiment No.	Sodium formate-C^{14}	L-Methionine-methyl-C^{14}	Folic acid	Aminopterin	Non-saponifiable		Sterols	
					Total activity $\times 10^{-3}$	Per cent	Total activity $\times 10^{-3}$	Per cent
1a	−	+	−	−	48	36	43	29
1b	−	+	+	−	33	22	29	19
2a	+	−	−	−	15	10	12	8
2b	+	−	+	−	26	17	22	15
3a	−	+	−	−	35	23	26	17
3b	−	+	−	+	59	39	54	36
4a	+	−	−	−	15	10	12	8
4b	+	−	−	+	3.5	2	1.5	1

The same conditions were used as in the experiment described in Table I. 0.5 μc. (0.5 μmole) of formate or methionine was added to each incubation. 40 mmoles of DL-homocysteine were added to each flask containing sodium formate-C^{14}.

TABLE III

Serine Experiments

Source of C^{14}	Additions	Sterols	
		Total activity $\times 10^{-3}$	Per cent
DL-Serine-3-C^{14}		7.4	4.9
"	L-Methionine	2.2	1.5
"	Sodium formate	2.9	1.9
L-Methionine-methyl-C^{14}		28.9	19.3
"	DL-Serine	77.0	51.3
Sodium formate-C^{14}		3.0	2.0
" "	DL-Serine	3.0	2.0
L-Methionine-methyl-C^{14}		29.4	19.6
"	DL-Serine	43.0	28.7
"	" + Aminopterin	57.7	38.4

The usual yeast homogenate was used. 48 hour incubation. 0.5 μc. of C^{14} per flask. Additions, where indicated, were methionine, 5 mg.; sodium formate, 5 mg.; serine, 5 mg.; Aminopterin, 2 mg.

Incubation with Doubly Labeled Methionine—A mixture of DL-methionine-methyl-C^{14} and DL-methionine-methyl-T, containing 0.72 mc. of C^{14} per

mmole and 2.1 mc. of T per mmole, was prepared. The usual yeast homogenate was used in two parallel experiments of five flasks each. After the incubation, the contents of the five flasks of each experiment were pooled. A total of 100 mg. of carrier ergosterol were added in each experiment and the mixtures were hydrolyzed, extracted with ether, and washed with KOH as before. The non-saponifiable matter was chromatographed over alumina, and the ergosterol fractions were further purified through precipitation with digitonin. The recovered ergosterol was recrystallized to constant specific activity. Purified ergosterol from each experiment was used for the measurement of the $T:C^{14}$ ratio.

Samples of the doubly labeled methionine and ergosterol were transferred to four counter cells each, dissolved in 2 ml. of methanol, and diluted with 18 ml. of 0.4 per cent solution of 2,5-diphenyloxazole in toluene. The

TABLE IV

Tritium to C^{14} Ratios in Methionine and Ergosterol

Experiment No.	T:C^{14}	
	DL-Methionine	Ergosterol
1	1.12 ± 0.06	0.97 ± 0.06
2	1.12 ± 0.06	1.02 ± 0.02

Each experiment consisted of five flasks, each flask containing 4 ml. of yeast extract in 1 per cent Tris-HCl buffer (pH 6), 5 mg. of serine, 2 mg. of Aminopterin, 1 mg. of ATP, and 0.175 mg. of doubly labeled methionine. Incubation time was 48 hours at room temperature. Radioactivities were assayed on a Packard Tri-Carb scintillation counter. The values given are the averages of four samples. For details of the isolation and purification of ergosterol, see the text.

samples were assayed in a Packard Tri-Carb scintillation counter, with use of the discriminator ratio method recommended by the Packard Instrument Company. The photomultiplier voltage was set at 1020 volts which provided the optimal contrast ratio between counts registered in the upper and lower channels without appreciable loss in counting efficiency in either channel. The discriminator controls were set at 10 to 50 volts for the lower channel and 50 to 100 volts for the upper channel. The samples were counted and the readings in both channels used in solving simultaneous equations to obtain two figures, one for the activity due to C^{14} in the upper channel and the other for the activity due to tritium in the lower channel. Since absolute activities (decompositions per minute) were not necessary to determine whether the ratios of $T:C^{14}$ in the starting material and in the products were the same, no efficiency factors were used and only observed counts per minute employed. The results in each series were averaged and the ratios of T to C^{14} were calculated (Table IV).

DISCUSSION

In aliquots of the same homogenate, methionine is converted into ergosterol four times more efficiently than formic acid (Table I). Addition of non-radioactive methionine suppresses the incorporation of formate-C^{14} into the sterol, while non-radioactive formate barely affects the incorporation of methionine-methyl-C^{14}. Homocysteine nearly doubles the incorporation of formate-C^{14} and addition of folic acid further increases the yield. On the other hand, Aminopterin sharply decreases the incorporation of formate-C^{14}.

Formate and homocysteine give methionine in biological systems in the presence of folic acid (6), and this fact may account for the increased incorporation of formate in the presence of this vitamin.

While formate alone does not significantly change the yield from methionine-methyl-C^{14}, formate and homocysteine decrease it considerably. Folic acid strengthens the formate-homocysteine effect. Addition of Aminopterin, which prevents the condensation of formate and homocysteine, reverses the inhibition of the incorporation of C^{14} from methionine. The data recorded in Table II confirm the fact, discernible in Table I, that folic acid is essential to formate-C^{14} incorporation into ergosterol-28-C^{14}, but has a deleterious effect on methionine-methyl-C^{14} conversion to the sterol methyl group, and, conversely, that Aminopterin inhibits formate-C^{14} utilization but is beneficial in the case of methionine-methyl-C^{14}.

All these facts point to the sequence: formate $\xrightarrow[\text{(folic acid)}]{\text{homocysteine}}$ methionine $\rightarrow$ ergosterol. The alternative possibility that folic acid may be necessary for a direct condensation of formate with a C_{27} steroid precursor of ergosterol fails to account for the beneficial effect of homocysteine. Aminopterin inhibition of formate but not of methionine proves that formate is not an intermediate between methionine and ergosterol. The higher yields obtained from methionine-methyl-C^{14} in the presence of Aminopterin may be due to the fact that, by inhibiting oxidation of the methyl group to formate and CO_2, Aminopterin insures that a higher percentage of the methyl groups is available for transfer to the sterol, and by inhibiting the synthesis of non-labeled methionine prevents a dilution of the radioactivity.

The increase in methionine-C^{14} incorporation in the presence of serine proves that the 1-carbon unit derived from serine is not on the pathway from methionine to ergosterol. When methionine donates its methyl group in sterol synthesis, the homocysteine moiety is liberated. Serine and homocysteine are known to combine to cystathionine, which does not contribute to the 1-carbon unit pool, therefore serine facilitates transmethylation by removing one of the reaction products. These data tend to confirm similar findings of Berg (6) and Doctor *et al.* (7), who found cystathionine inactive as 1-carbon donor.

The final two experiments represent an attempt to prove that transmethylation has taken place. If 1 tritium atom were lost in the process, only 66 per cent of the tritium of methionine would appear in ergosterol. In the first experiment 86 per cent, and in the second experiment 91 per cent, of the tritium was found in the sterol.

Because of the possibility of isotope effects (8), these figures alone are insufficient to prove definitely that a transfer of an intact methyl group has taken place; they support, however, the conclusions reached from the series of experiments reported in Tables I and II. Together, this body of evidence leaves little doubt that the yeast is capable of a true transmethylation to carbon.

SUMMARY

1. Formate-C^{14} incorporation into ergosterol in yeast homogenates is inhibited by excess methionine, as well as by Aminopterin, a folic acid antagonist. It is stimulated by the addition of homocysteine and folic acid.

2. The incorporation of methionine-methyl-C^{14} is not affected by formate alone but is inhibited by formate, homocysteine, and folic acid. This inhibition is reversed by Aminopterin.

3. The incorporation of methionine-methyl-C^{14} is stimulated by Aminopterin and serine.

4. A mixture of methionine-methyl-C^{14} and methionine-methyl-T and the ergosterol resulting from it have the same T:C^{14} ratios.

5. These observations strongly support the conclusion that a hitherto unknown transmethylation has been found, namely the transfer of an intact methyl group from sulfur to carbon.

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STUDIES ON THE INHIBITORY NATURE OF PTERIDINES*

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(Received for publication, December 17, 1957)

Mallette and coworkers (1) were the first to synthesize 2,4-diaminopteridine and four derivatives of this type, namely the 6,7-dimethyl, diphenyl, phenanthro, and acenaphtho compounds. Daniel *et al.* (2) and Daniel and Norris (3) reported strong inhibitory properties of these compounds for the growth of *Streptococcus faecalis* and *Lactobacillus casei* which require preformed pteroylglutamic acid (PGA). These workers further reported that the pteridines also inhibit the growth of *Lactobacillus arabinosus*, *Staphylococcus aureus*, and *Escherichia coli*, which do not require preformed PGA (2, 3). The hematologic effect in rats of some of the pteridines synthesized by Mallette *et al.* (1) is reported by Swendseid *et al.* (4), who fed 2,4-diamino-6,7-diphenylpteridine to rats at 50 mg. per cent and noted leucopenia without any reduction in hemoglobin in a 2 week test period. The other pteridines, when fed at the above level, had no hematologic effect. Growth inhibition of *L. casei* by low concentrations of 2,4-diamino-6,7-dimethylpteridine is competitively antagonized by PGA, but at high concentrations of the pteridine PGA fails to reverse the inhibition (5). Campbell *et al.* (6, 7) have synthesized about 70 2,4-diaminopteridines with various substitutions in the 6 and 7 positions. These pteridines are reported (8) to inhibit the growth of *Leuconostoc citrovorum*, *S. faecalis*, *Streptococcus pyogenes*, *S. aureus*, *Vibrio cholerae*, and others. The inhibition of *L. citrovorum* at low concentrations of the 6,7-diisopropyl-2,4-diaminopteridine is competitively reversed by citrovorum factor (CF), but at high concentrations of the pteridine the inhibition ceases to be competitive and becomes irreversible by CF (9).

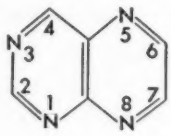
Spickett and Timmis (10) have reported the synthesis of a number of 2,4,7-triamino-6-arylpteridines, some of which showed antifolic acid activity. Continuing this work, Osdene and Timmis (11, 12) prepared a number of related pteridines which carry functional groups in the 6 position

* This work was supported by grants from the National Cancer Institute, National Institutes of Health, United States Public Health Service, Bethesda, Maryland. Leucovorin (Lederle), folic acid, and Aminopterin were generously supplied by Dr. H. P. Broquist of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York. Pteridines were obtained through the courtesy of Dr. T. S. Osdene of the Department of Biochemistry at this institution.

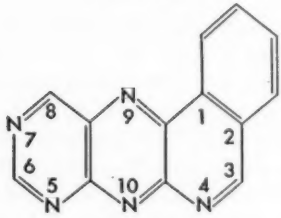
and also examples of some allied ring systems (13). The present work concerns the study of the inhibitory properties of some of these and four new pteridines in various biological systems.

EXPERIMENTAL

The pteridines used in the present studies have the accompanying basic structures.



pteridine



4,5,7,9,10-penta-aza-1,2-benzanthracene

Effect of 2,4-Diaminopteridines and 4,7-Diaminopteridines on Conversion of PGA to CF by Chick Liver Supernatant Solution—Straight run (New Hampshire males with single comb white Leghorn females) cross-bred chicks of 10 to 12 weeks of age were used for the source of liver throughout the experiments. The diet used consisted of Purina Startena (Ralston Purina Company), supplemented by 10 mg. of PGA and 30 γ of vitamin B_{12} per kilo of diet. The chick liver supernatant solutions were prepared in the following manner: 20 per cent homogenates of chick liver were made in 0.08 M sodium potassium phosphate buffer, pH 6.6, with a Waring blendor. An aliquot of the homogenate was centrifuged for 10 minutes at 13,000 $\times$ g and the clear portion of the supernatant solution was decanted off and made to the volume of the starting homogenate with an aliquot of the same buffer. 5 ml. each of the supernatant solution were tested for PGA to CF conversion studies as described in Table I. At the end of the incubation period, the incubation mixtures were neutralized to pH 6.8 and autoclaved for 30 minutes at 120°. After cooling, the samples were homogenized in a Waring blendor, made up to volume, filtered, and the filtrate was assayed. The CF content of the liver samples

was determined by using the Bacto-CF assay medium (Difco Laboratories Inc., Detroit, Michigan). *L. citrovorum* ATCC 8081[1] was used as the test organism, and leucovorin (Lederle) was employed as the standard.

TABLE I

Effect of 2,4-Diamino- and 4,7-Diamino-Substituted Pteridines on Conversion of PGA to CF by Chick Liver Supernatant Solutions

Additions	CF per gm. liver
2,4-Diaminopteridines	
	γ
Complete system*	57
" " + 2,4-diamino-7-hydroxypteridine-6-carboxylic acid	25
" " + 2,4,7-triaminopteridine	23
" " + 2,4,7-triaminopteridine-6-carboxylic acid	49
" " + 2,4,7-triaminopteridine-6-carboxyamide	27
" " + 2,4,7-triaminopteridine-6-*N*-methylamide	15
" " + 2,4,7-triamino-6-*o*-methylphenylpteridine	4
" " + 3,6,8-triamino-4,5,7,9,10-pentaaza-1,2-benzanthracene	11
Complete system without PGA	4
4,7-Diaminopteridines	
Complete system*	77
" " + 4,7-diaminopteridine	80
" " + 4,7-diaminopteridine-6-carboxylic acid	66
" " + 4,7-diaminopteridine-6-carboxyamide	73
" " + 4,7-diaminopteridine-6-*N*-methylamide	68
" " + 4,7-diamino-2-methylthiopteridine-6-carboxylic acid	82
Complete system + 4,7-diamino-2-methylthiopteridine-6-carboxyamide	80
Complete system without PGA	4

* Complete system: 5 ml. of liver supernatant solutions in 0.08 M phosphate buffer of pH 6.6, along with 5 ml. of the same buffer and aliquots of solutions containing the following amounts of substances to make a total volume of 11 ml.: 100 γ of PGA, 10 mg. of serine, 5 mg. of homocysteine, 10 mg. of magnesium chloride, and 1 mg. of the pteridines. Flasks were incubated under nitrogen at 37° for 3 hours and were later autoclaved at 120° for 30 minutes.

The cultures were incubated for 48 hours at 37° and growth was measured by turbidimetric procedure. Since leucovorin has been reported to be

[1] A report by Felton and Niven (14) concerning a taxonomic study on the culture *L. citrovorum* ATCC 8081 suggests that the latter organism is a typical strain of *Pediococcus cerevisiae*, as described earlier by Pederson (15).

half as active as the CF isolated from a horse liver (16), the microbiological assay values are divided by 2 in order to express the results in terms of the naturally occurring CF.

Microbiological Experiments—The inhibitory property of 2,4,7-triamino-6-*o*-methylphenylpteridine was studied with *S. faecalis* R and *L. citrovorum* 8081 as the test organisms. The former was grown on PGA assay medium (17) for 18 hours in the presence of different levels of PGA, and the *L. citrovorum* was grown on Bacto-CF assay medium (Difco Laboratories, Inc., Detroit, Michigan) for 48 hours in the presence of different levels of leucovorin (Lederle).

Experiments with Rats—Adult male rats of the Sprague-Dawley strain were used in the present experiment. Rats were maintained on a PGA-deficient diet (Nutritional Biochemicals Corporation, Cleveland 28, Ohio) and the effect of supplementing this diet with Aminopterin, 2,4,7-triamino-6-*o*-methylphenylpteridine, PGA, and CF on growth and white blood count was studied.

RESULTS AND DISCUSSION

It is apparent from the results of Table I that addition of 2,4-diamino-7-hydroxypteridine-6-carboxylic acid inhibits the conversion of PGA to CF by 50 per cent. It is interesting to note the different results obtained by varying a functional group in the 6 position. 2,4,7-Triaminopteridine itself inhibits CF synthesis from 57 to 23 γ while the corresponding 6-carboxylic acid is without effect. On the other hand, the inhibition by 6-carboxyamide compares with that of the 6-unsubstituted pteridine, and the 6-*N*-methylamide is even more inhibitory than the 6-carboxyamide. The two most effective inhibitors are 2,4,7-triamino-6-*o*-methylphenylpteridine and 3,6,8-triamino-4,5,7,9,10-pentaaza-1,2-benzanthracene. Addition of 1 mg. quantity of each of the 4,7-diaminopteridines tested had no significant inhibitory effect. These results suggest that only the derivatives of condensed pyrimidine system which contain the 2,4-diamino moiety in the pyrimidine ring inhibit the enzyme system responsible for the conversion of PGA to CF. Since 1 mg. of 2,4,7-triamino-6-*o*-methylphenylpteridine completely blocked the enzymatic conversion of 100 γ of PGA to CF, it was interesting to study the relationship, if any, between the inhibitory effects of this and other structurally related pteridines. Studies were conducted to compare the inhibitory effect of 2,4,7-triamino-6-phenylpteridine and the three methyl-substituted isomers, *viz.* 2,4,7-triamino-6-*o*-methylphenylpteridine, 2,4,7-triamino-6-*m*-methylphenylpteridine, and 2,4,7-triamino-6-*p*-methylphenylpteridine. The results are presented in Table II. It is apparent from the results that 2,4,7-triamino-6-*o*-methylphenylpteridine is the most effective inhibitor. In contrast to this, a methyl group in the para position of the phenyl

ring is detrimental to the inhibitory property of the pteridine and the meta methylphenyl and the unsubstituted phenyl compounds are less inhibitory than the ortho methylphenyl isomer.

TABLE II

Inhibitory Effect of 2,4,7-Triamino-6-phenylpteridines on Conversion of PGA to CF by Chick Liver Supernatant Solutions

Inhibitor	Inhibition index*
2,4,7-Triamino-6-phenylpteridine	0.11
2,4,7-Triamino-6-*o*-methylphenylpteridine	0.04
2,4,7-Triamino-6-*m*-methylphenylpteridine	0.15
2,4,7-Triamino-6-*p*-methylphenylpteridine	5.80

* In each instance varying concentrations of inhibitor were added to the complete system (described under Table I) and the amount of CF synthesized from 100 γ of PGA was measured for each level of the inhibitor. An inhibition curve was then plotted from which the point of half maximal inhibition was obtained. The ratio of the amount of inhibitor to PGA that will produce half maximal inhibition is defined as the inhibition index.

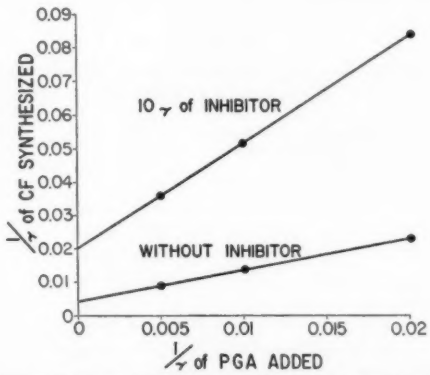


FIG. 1. Inhibition of PGA to CF conversion by 2,4,7-triamino-6-*o*-methylphenylpteridine. The enzyme system was the same as that described under Table I. Quantities of CF synthesized from various levels of PGA added were measured with or without the presence of the pteridine.

The conversion of PGA to CF under these experimental conditions is reported to be inhibited by Aminopterin, 5-substituted 2,4-diaminopyrimidine, and 2,6-diaminopurine (18).

Enzyme kinetic studies were conducted in order to determine whether the inhibition of PGA to CF conversion by 2,4,7-triamino-6-*o*-methylphenylpteridine is competitive or non-competitive (Fig. 1). The plot of the reciprocal of PGA concentrations against the reciprocal of CF synthe-

sized in the presence and in the absence of the pteridine gives two separate straight lines with different slopes and intercepts. According to the

TABLE III

Effect of Pteridines on Conversion of THPGA to CF by Chick Liver Supernatant Solutions

Additions	CF synthesized
	γ per gm. liver
Complete system*	96
" " + 2,4,7-triamino-6-*o*-methylphenylpteridine	89
" " + 3,6,8-triamino-4,5,7,9,10-pentaaza-1,2-benzanthracene	80

* Complete system: 5 ml. of supernatant solutions of liver in 0.08 M phosphate buffer, pH 6.6, along with the same buffer and aliquots of solutions containing the following substances to make a total volume of 11 ml.: 200 γ of THPGA, 5 mg. of homocysteine, 10 mg. of serine, 10 mg. of magnesium chloride, 1 mg. of 2,4,7-triamino-6-*o*-methylphenylpteridine, and 10 mg. of 3,6,8-triamino-4,5,7,9,10-pentaaza-1,2-benzanthracene. The flasks were incubated under nitrogen at 37° for 3 hours and were later autoclaved at 120° for 30 minutes.

TABLE IV

Effect of PGA and 2,4,7-Triamino-6-o-methylphenylpteridine on Growth of S. faecalis R

PGA	2,4,7-Triamino-6-*o*-methylphenylpteridine	Optical density	Inhibition index*
mμgm.	*mμgm.*		
0.0		0.00	
0.5		0.16	
1.0		0.37	
1.5		0.50	
2.0		0.52	
2.5		0.59	
3.0		0.63	
3.5		0.65	
4.0		0.66	
4.0	1,000	0.43	315
4.0	2,000	0.17	
4.0	5,000	0.02	
40.0	2,000	0.56	165
40.0	5,000	0.41	
40.0	10,000	0.22	
400.0	10,000	0.33	25
400.0	25,000	0.18	
400.0	125,000	0.03	

* Ratio of the amount of 2,4,7-triamino-6-*o*-methylphenylpteridine to PGA that will produce half maximal growth.

mathematical formulation of Lineweaver and Burk (19), this would indicate a non-competitive relationship between PGA and 2,4,7-triamino-6-*o*-methylphenylpteridine for the converting enzyme.

Since tetrahydropteroylglutamic acid (THPGA) has been postulated as the intermediate before formylation in the conversion of PGA to CF (20–22), it was of interest to determine whether the conversion of PGA to

TABLE V

Effect of CF and 2,4,7-Triamino-6-o-methylphenylpteridine on Growth of L. citrovorum 8081

CF*	2,4,7-Triamino-6-*o*-methylphenylpteridine	Optical density	Inhibition index†
mμgm.	mμgm.		
0.0		0.00	
0.1		0.06	
0.2		0.15	
0.3		0.21	
0.4		0.26	
0.5		0.29	
0.6		0.30	
0.7		0.30	
0.8		0.31	
1.0		0.32	
1.0	1,000	0.28	2400
1.0	2,500	0.25	
1.0	5,000	0.15	
1.0	7,500	0.10	
10.0	12,500	0.30	2150
10.0	25,000	0.25	
10.0	37,500	0.17	
10.0	50,000	0.13	
10.0	75,000	0.08	

* Since leucovorin (Lederle) is only half as active as the naturally occurring CF, 2 mμgm. of leucovorin were added per millimicrogram of CF.

† Ratio of the amount of 2,4,7-triamino-6-*o*-methylphenylpteridine to CF that will produce half maximal growth.

THPGA was inhibited by 2,4,7-triamino-6-*o*-methylphenylpteridine and 3,6,8-triamino-4,5,7,9,10-pentaaza-1,2-benzanthracene in the present enzyme system. The results presented in Table III indicate that the addition of 1 mg. of 2,4,7-triamino-6-*o*-methylphenylpteridine or 10 mg. of 3,6,8-triamino-4,5,7,9,10-pentaaza-1,2-benzanthracene do not inhibit the conversion of THPGA to CF. Since these two analogues are reported in Table I to inhibit significantly the conversion of PGA to CF, the results would suggest that the analogues may inhibit the conversion of PGA to THPGA.

The data on the inhibition of *S. faecalis* R by 2,4,7-triamino-6-*o*-methylphenylpteridine and its reversal by different levels of PGA are summarized in Table IV. The fact that increasing amounts of the PGA can reverse the effect of higher levels of the analogue suggests that the analogue may be affecting some enzyme system which is directly concerned in the metabolism of PGA. It is also interesting to note that the inhibition is not truly of a competitive nature as shown by the inconstancy of the inhibition index. The data in Table V suggest that the growth of *L. citrovorum* 8081 is inhibited by 2,4,7-triamino-6-*o*-methylphenylpteridine

TABLE VI

Effect of 2,4,7-Triamino-6-o-methylphenylpteridine, Aminopterin, PGA, and CF on Growth and White Blood Count of Rats

Group No.	Supplement to basal diet,* given intraperitoneally per day per rat	Average weight†		Average white blood count,† cells per c.mm. × 10^{-3}		
		Initial	4 wks.	Initial	1 wk.	4 wks.
		gm.	*gm.*			
1	None	268 (8)	330 (8)	16.4 (8)	16.6 (8)	18.3 (8)
2	25 γ Aminopterin	264 (8)		16.4 (8)	11.2 (8)	
3	5 mg. 2,4,7-triamino-6-*o*-methylphenylpteridine	261 (8)	304 (7)	21.0 (8)	17.1 (8)	12.5 (7)
4	5 mg. 2,4,7-triamino-6-*o*-methylphenylpteridine plus 200 γ PGA	261 (8)	311 (7)	19.2 (7)	14.4 (7)	21.5 (7)
5	5 mg. 2,4,7-triamino-6-*o*-methylphenylpteridine plus 100 γ CF	263 (8)	311 (8)	19.7 (8)	19.9 (8)	23.3 (8)

* Rats were fed a folic acid-deficient diet (Nutritional Biochemicals Corporation, Cleveland 28, Ohio).

† The figures in parentheses indicate the number of rats alive.

and that CF reverses the inhibitory effect of the analogue in a competitive manner. Derivatives of 2,4-diaminopteridine are reported (2, 3) to inhibit the growth of *S. faecalis* and *L. casei* which require PGA, and also the growth of *L. arabinosus*, *S. aureus*, and *E. coli*, which do not require PGA. The growth of *L. citrovorum* is inhibited by 6,7-diisopropyl-2,4-aminopteridine and the inhibition is reversed by CF only at low concentrations of the analogue (9). The inhibitory effect of Aminopterin on the growth of *S. faecalis* is reported to be reversed non-competitively by PGA (23) while CF competitively reverses the Aminopterin inhibition of *L. citrovorum* (24).

Studies on the effect of 2,4,7-triamino-6-*o*-methylphenylpteridine on white blood count and growth of rats are described in Table VI. The

animals in Group 2 were leucopenic at the end of the 1st week. This was followed by loss of appetite, diarrhea, hemorrhage around the eyelids, and death before the end of the 2nd week. Daily administration of 5 mg. of 2,4,7-triamino-6-*o*-methylphenylpteridine per rat (Group 3) for a period of 1 month produces leucopenia without loss in weight or other toxic effects. One rat in this group died at the end of the 3rd week with a white blood count of 5.7 thousand cells per c.mm. The results of white blood counts in rats of Group 4 indicate an initial drop from 19.2 to 14.4 thousand cells per c.mm. at the end of the 1st week, which is reversed at the end of 4 weeks, at which time the white blood count is 21.5 thousand cells per c.mm. It is apparent from the results that CF (Group 5) effectively reverses the leucopenic effect of the analogue throughout the experiment and that PGA reverses but slowly.

SUMMARY

Several 2,4,7-triaminopteridines significantly inhibit the enzymatic conversion of pteroylglutamic acid (PGA) to citrovorum factor (CF) by chick liver supernatant solutions, while all the 4,7-diaminopteridines tested were without effect. The most effective inhibitor among the pteridines tested was 2,4,7-triamino-6-*o*-methylphenylpteridine. Enzyme kinetic studies indicate a non-competitive relationship between PGA and the above analogue for the converting enzyme. Since the analogue did not inhibit the conversion of tetrahydropteroylglutamic acid (THPGA) to CF, it may interfere with the reduction of PGA to THPGA.

2,4,7-Triamino-6-*o*-methylphenylpteridine inhibits the growth of *Streptococcus faecalis* and *Leuconostoc citrovorum* 8081. The inhibitory effect of the analogue on the growth of the former organism is reversed non-competitively by PGA while CF reverses the inhibition of the latter organism in a competitive manner.

Daily administration of 5 mg. of 2,4,7-triamino-6-*o*-methylphenylpteridine to rats for a 1 month period causes leucopenia, which is reversed by the simultaneous administration of 200 γ of PGA per day or 100 γ of CF per day.

The author is indebted to Dr. Tad Patton for preparing tetrahydropteroylglutamic acid and to Dr. T. S. Osdene for preparing 2,4,7-triamino-6-phenylpteridine and the three methyl substituted isomers used in the present study. Technical assistance of Mrs. Peggy Dixon and Mr. William B. Kitzman is gratefully acknowledged.

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THE SYNTHESIS OF CELLULOSE IN CELL-FREE EXTRACTS OF ACETOBACTER XYLINUM*

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(Received for publication, December 23, 1957)

In a previous publication (2) evidence was presented that the synthesis of chitin in *Neurospora crassa* occurs by way of a glycosyl transfer from uridine diphosphate-*N*-acetylglucosamine. The similarity of the structure of chitin and cellulose suggested that a like mechanism may be operative in the biosynthesis of cellulose. It has been shown by Hestrin and Schramm (3) that non-viable lyophilized preparations of *Acetobacter xylinum* will produce cellulose from glucose in the presence of oxygen. While the work reported here was in progress, the preparation of a cell-free extract of *A. xylinum* capable of synthesizing cellulose from glucose and adenosine triphosphate was reported by Colvin (4) and also by Greathouse (5). Evidence will be presented in this communication that the glycosyl donor in the synthesis of cellulose by a cell-free preparation of *A. xylinum* is uridine diphosphoglucose.[1]

EXPERIMENTAL

Materials and Methods—C^{14}-Glucose 6-phosphate was prepared from uniformly labeled C^{14}-glucose[2] with yeast hexokinase and adenosine triphosphate (Sigma Chemical Company) and crystallized as the barium salt (6). C^{14}-Glucose 1-phosphate was prepared from C^{14}-labeled starch with muscle phosphorylase and amylo-1,6-glucosidase[3] and crystallized as the K salt (7).

C^{14}-UDPG was prepared from glucose 6-phosphate and uridine triphosphate (Sigma Chemical Company) with a crude yeast enzyme containing both phosphoglucomutase and UDPG-pyrophosphorylase (8, 9). It was purified by chromatography on Dowex 1 formate, essentially by the pro-

* This work, on which a preliminary report has been published (1), has been supported by a grant (No. RG-4761) from the National Institutes of Health, United States Public Health Service.

† Research Fellow of the Helen Hay Whitney Foundation.

[1] The following abbreviations are used: uridine diphosphoglucose, UDPG; glucose 1-phosphate, G-1-P; tris(hydroxymethyl)aminomethane, Tris; ethylenediaminetetraacetic acid, EDTA; uridine diphosphate, UDP.

[2] Isotopes obtained on allocation from the Atomic Energy Commission.

[3] Kindly made available by Dr. B. Illingworth.

cedure of Hurlbert *et al.* (10), followed by adsorption on charcoal and elution with 50 per cent ethanol-1 per cent NH_4OH (v/v). The radioactive UDPG was diluted to the desired specific activity with UDPG obtained from the Sigma Chemical Company.

A high molecular weight, mixed cellodextrin fraction was prepared by the procedure of Zechmeister and Toth (11) and corresponds to the water-soluble Fractions A and B of these authors.

A partially purified cellulase from *Aspergillus niger* was prepared from commercial cellulase (Nutritional Biochemicals Corporation)[4] which converted cellodextrins to glucose; it still retained significant maltase activity.

Purified cellulase from *Myrothecium verrucaria* was a gift from Dr. D. R. Whitaker. The enzyme was free from maltase activity, and hydrolyzed cellodextrins to a mixture of glucose and cellobiose; cellobiose was hydrolyzed very slowly by this enzyme (12, 13).

A. xylinum, ATCC 10821, was grown and freed from cellulose as described by Hestrin and Schramm (3). 6 to 8 ml. of packed cells were suspended in 25 ml. of 0.05 M Tris-0.01 M $MgCl_2$-0.001 M EDTA, pH 7.5, and ruptured by treatment for 30 minutes in a 9 kc. Raytheon magnetostriction oscillator. The cell debris was removed by centrifugation at 15,000 × *g* for 12 minutes and the turbid supernatant fluid was centrifuged at 140,000 × *g* for 1 hour. The pellet was resuspended in the same buffer with a TenBroeck homogenizer and recentrifuged. The resulting pellet was suspended in 8 ml. of buffer and could be kept frozen for several weeks without loss in activity.

For enzymatic assay the enzyme was incubated as indicated for the individual experiments: 10 mg. of cellulose powder (Whatman) were added at the end of the incubation and the reaction was stopped by heating at 100° for 5 minutes. After cooling, the tubes were centrifuged at 2000 × *g* and the precipitate of denatured protein and cellulose was washed with 2 ml. of water by centrifugation.

The precipitate was then suspended in 2 ml. of 1 per cent NaOH (identical results were obtained when 4 per cent NaOH was used) and heated at 100° for 5 minutes, cooled, and diluted to 5 ml. The insoluble cellulose was collected by centrifugation and washed three times with 2 ml. of water. Radioactivity was determined in suitable aliquots in a proportional gas flow counter (Nuclear Instrument and Chemical Corporation). The results were reproducible within 10 per cent between duplicate incubation mixtures.

Enzymatic Synthesis of Cellulose from UDPG—Incubation of the particulate fraction of *A. xylinum* with C^{14}-UDPG and cellodextrins gave rise to a radioactive, water-insoluble, alkali-insoluble material (Table I). Neither C^{14}-G-1-P nor C^{14}-glucose could substitute for UDPG in this sys-

[4] Glaser, L., unpublished procedure.

tem. No radioactivity was present in the unincubated controls. Table II shows that the radioactivity in the cellulose was not decreased by the

TABLE I

Enzymatic Synthesis of Cellulose from UDPG

The reaction mixtures contained 12 mg. of cellodextrins, 80 μmoles of Tris, 7 μmoles of $MgCl_2$, 0.7 μmole of EDTA, 0.7 ml. of enzyme, and radioactive substrate as indicated, in a final volume of 1.8 ml.; pH 8.2; 28°. The data below have been published previously (1).

Radioactive substrate			Time of incubation	C^{14} in cellulose
	μmoles	*c.p.m.*	*min.*	*c.p.m.*
UDPG	3.5	83,500	0	6.2
"	3.5	83,500	120	1220
G-1-P	5.1	87,500	120	22
Glucose	7.1	78,000	120	0

TABLE II

Effect of Addition of Various Sugars on Cellulose Synthesis

The reaction mixtures contained 12 mg. of cellodextrins, 80 μmoles of Tris, 7 μmoles of $MgCl_2$, 0.7 μmole of EDTA, and 0.7 ml. of enzyme in a final volume of 1.8 ml.; pH 8.2; 28°. In Experiments A, B, and C, the reaction mixture contained 4.1 μmoles of C^{14}-UDPG (40,000 c.p.m. per μmole); in Experiment D, 6 μmoles of C^{14}-UDPG (14,000 c.p.m. per μmole). Different enzyme preparations were used in Experiments A, B, C, and D.

Experiment No.	Additions	Time of incubation	C^{14} in cellulose
		min.	*c.p.m.*
A	None	0	6
	"	120	300
	Maltose, 4 μmoles	120	324
	Sucrose, 4 "	120	294
B	None	120	336
	Cellobiose, 4 μmoles	120	390
C	None	120	765
	Glucose, 4 μmoles	120	714
D	None	120	402
	Trehalose, 4 μmoles	120	383

presence in the reaction mixtures of unlabeled glucose, maltose, sucrose, cellobiose, or trehalose.

To identify this material, a large scale incubation similar to that in Table I was carried out, and the isolated material (25 mg., 4450 c.p.m.) was partially hydrolyzed by being dissolved in HCl saturated at 0° and by allowing the solution to stand for 4 hours at 20°. The HCl was removed

by repeated evaporation under reduced pressure. The material was finally suspended in water and the remaining insoluble material discarded. 2750 c.p.m. were recovered in the soluble fraction.

Descending paper chromatography of this solution on Whatman No. 1 paper, with butanol-pyridine-water (6:4:3) (14) as the solvent, revealed, after elution, radioactive materials with the mobility of glucose and cello-

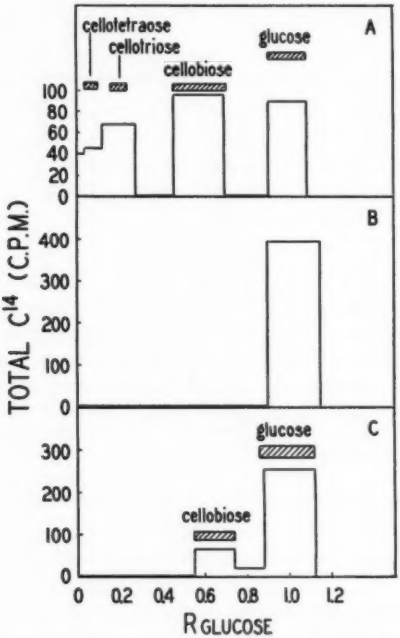

FIG. 1. Paper chromatogram of partial acid digest of C^{14}-cellulose. The details are given in the text. Cross-hatched areas show the position of known standards revealed by benzidine-trichloroacetic acid spray for reducing substances (15). In Chromatograms A and C the glucose had moved 32 cm. from the origin; in Chromatogram B, 38 cm.

biose and slower moving materials occupying the position of cellotriose and cellotetraose and higher molecular weight compounds (Fig. 1, *A*). Pooled eluates of the cellobiose region were crystallized (16) after the addition of cellobiose carrier. The data in Table III show that the radioactivity remained essentially constant after four crystallizations.

The partial acid digest was digested further with *A. niger* cellulase. After chromatography, the only radioactive material had the mobility of glucose (Fig. 1, *B*).

When the partial acid digest was treated with *M. verrucaria* cellulase and chromatographed (Fig. 1, *C*), only two radioactive areas, those corresponding to glucose and cellobiose, remained, which is consistent with the observation (13) that this enzyme will split cellobiose at only a very slow rate.

These data are consistent with the assumption that the radioactive material is present in a glucose polymer having β-1,4 linkages, which is the basic structure of cellulose. The chain length of the polymer is unknown.

Properties of Enzyme—The enzyme was routinely assayed at pH 8.3, the rate of the reaction decreasing at a lower pH. At pH 7.5, the rate is 39 per cent of that at pH 8.3, and at pH 6.5 it is 13 per cent of the former rate. The enzyme was not tested under more alkaline conditions because of the instability of UDPG (17). Although no dependence on Mg^{++} could be demonstrated, this divalent ion was added to all the incubation mixtures.

TABLE III

Crystallization of Cellobiose

For the details, see the text.

Crystallization No.	Total cellobiose crystallized	Cellobiose counted	C^{14} in cellobiose
	mg.	*mg.*	*c.p.m. per mg.*
1	7.5	0.75	36
2	5.25	1.05	44.0
3	3.5	1.20	35
4	1.8	0.75	45.4

The effect on cellulose synthesis of varying the time of incubation, the enzyme concentration, and the UDPG concentration is shown in Figs. 2, 3, and 4, respectively.

The synthesis of cellulose is stimulated by the addition of cellodextrins to the reaction mixture (Fig. 5). With different preparations the stimulation varies between 5- and 20-fold. A cellodextrin solution containing 80 mg. per ml. was further fractionated with ethanol. The fractions precipitating between 0 and 30 per cent ethanol (I) and 30 and 70 per cent alcohol (II) were tested in the enzyme system (Fig. 5, *B*). Fraction I, which contains cellodextrins of a higher molecular weight, shows higher activity per unit weight. By analogy with muscle phosphorylase (18) and chitin synthetase (2), the cellodextrin stimulation may be the result of a primer action, and the variable rates observed in the absence of added primer may be due to the presence of cellodextrins in the enzyme preparation.

To establish primer dependence, the enzyme suspension was incubated with *M. verrucaria* cellulase (0.2 mg. per ml. for 30 minutes at pH 7.5).

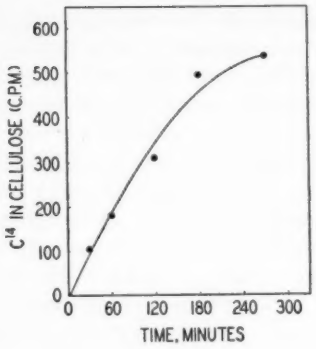

FIG. 2

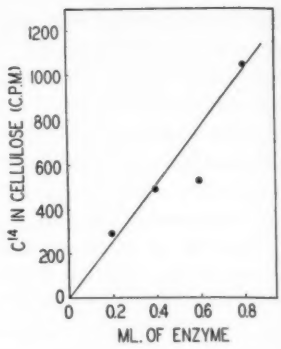

FIG. 3

FIG. 2. Cellulose synthesis as a function of time. The reaction mixture contained 12 mg. of cellodextrins, 4.1 μmoles of C^{14}-UDPG (40,500 c.p.m. per μmole), 70 μmoles of Tris, 6 μmoles of $MgCl_2$, 0.6 μmole of EDTA, and 0.6 ml. of enzyme in a final volume of 1.7 ml.; pH 8.3; 29°.

FIG. 3. Cellulose synthesis as a function of enzyme concentration. The reaction mixture contained 12 mg. of cellodextrins, 80 μmoles of Tris, 8 μmoles of $MgCl_2$, 0.8 μmole of EDTA, and 7.2 μmoles of C^{14}-UDPG (15,800 c.p.m. per μmole) in a final volume of 2 ml.; pH 8.3. Incubation was for 2 hours at 29°.

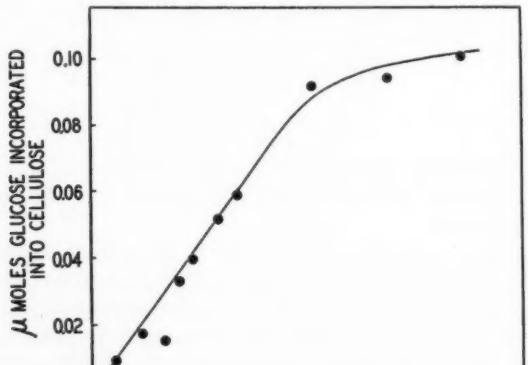

FIG. 4. Cellulose synthesis as a function of UDPG concentration. The reaction mixture contained 12 mg. of cellodextrins, 90 μmoles of Tris, 6 μmoles of $MgCl_2$, 0.6 μmole of EDTA, 0.6 ml. of enzyme, and UDPG as indicated (23,500 c.p.m. per μmole) in a final volume of 2.3 ml.; pH 8.3. Incubation was for 2 hours at 29°.

The cellulose-synthesizing enzyme was then reisolated by centrifugation at 140,000 × g for 1 hour, suspended in 0.05 M Tris-0.01 M $MgCl_2$-0.001

M EDTA, pH 7.5, and assayed. The data in Table IV indicate that treatment of the enzyme with cellulase essentially abolished the activity in the absence of added primer. Thus in Experiment B, before cellulase treatment, the ratio of activities in the presence and the absence of cellodextrins was 5.9:1; after cellulase treatment this ratio became 48:1.

While the dependence of cellulose synthesis on the presence of soluble primer suggested that a net synthesis of cellulose rather than an exchange

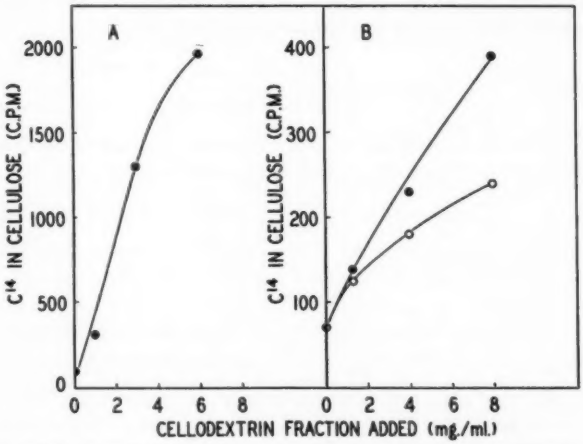

FIG. 5. Cellulose synthesis as a function of cellodextrin concentration. The reaction mixture contained the following. *A*, cellodextrins as indicated, 80 μmoles of Tris, 6 μmoles of $MgCl_2$, 0.6 μmole of EDTA, 3.5 μmoles of C^{14}-UDPG (20,000 c.p.m. per μmole), and 0.6 ml. of enzyme in a final volume of 1.8 ml.; pH 8.3. Incubation was for 2 hours at 29°. *B*, cellodextrins as indicated, 75 μmoles of Tris, 5 μmoles of $MgCl_2$, 0.5 μmole of EDTA, 4.1 μmoles of C^{14}-UDPG (40,000 c.p.m. per μmole), and 0.5 ml. of enzyme in a final volume of 1.6 ml.; pH 8.3. Incubation for 2 hours at 29°. ●, cellodextrin Fraction I; ○, cellodextrin Fraction II. Different enzyme preparations were used in *A* and *B*. The details are given in the text.

reaction was taking place, the following experiments were carried out to test this hypothesis (Table V). The enzyme was incubated with cellodextrins and C^{14}-UDPG. After incubation for 1 hour, the enzyme and the cellulose formed were isolated by centrifugation and washed, and various aliquots were reincubated as indicated in Table V. Experiment D shows that the enzyme still retained activity, and in Experiments B and C, the radioactivity in the cellulose was not decreased by incubation with non-radioactive UDPG, in the presence or the absence of primer. Experiment E shows that the radioactivity in the cellulose was unaffected by incubation with UDP.

Some soluble cellodextrins are also formed during the enzymatic syn-

thesis of insoluble cellulose. If, after deproteinization, the supernatant fluid is freed from C^{14}-UDPG and other ionic material by passage through an Amberlite MB-2 resin column (2) and then digested with *M. verrucaria*

TABLE IV

Cellodextrin Stimulation in Cellulase-Treated Enzyme

The reaction mixtures contained 80 μmoles of Tris, 8 μmoles of $MgCl_2$, 0.8 μmole of EDTA, 7 μmoles of C^{14}-UDPG (12,400 c.p.m. per μmole), and 0.8 ml. of enzyme in a final volume of 1.9 ml.; pH 8.3. Incubation was for 2 hours at 29°. Different enzyme preparations were used in Experiments A and B.

Experiment No.	Cellodextrins added	C^{14} in cellulose	
		Original enzyme	Cellulase-treated enzyme
	mg.	*c.p.m.*	*c.p.m.*
A	0	123	21
	12	194	168
B	0	192	22.5
	12	1140	1008

TABLE V

Non-Exchange of Glucose between UDPG and Insoluble Cellulose

The initial reaction mixture contained 80 mg. of cellodextrins, 30 μmoles of C^{14}-UDPG (30,000 c.p.m. per μmole), 500 μmoles of Tris, 45 μmoles of $MgCl_2$, 4.5 μmoles of EDTA, and 4.5 ml. of enzyme in a final volume of 12.5 ml.; pH 8.3. Incubation was for 1 hour at 29°. After incubation, the solution was centrifuged for 1 hour at 140,000 × *g*. The pellet was washed by centrifugation with 0.05 M Tris-0.01 M $MgCl_2$-0.001 M EDTA, pH 7.5, and finally suspended with a TenBroeck homogenizer in 6 ml. of the same buffer and used in the second incubation, which contained UDPG and cellodextrins as indicated, 120 μmoles of Tris, 12 μmoles of $MgCl_2$, 1.2 μmoles of EDTA, and 1.2 ml. of the enzyme in a final volume of 2.1 ml.; pH 8.3; 29°.

Experiment No.	Additions	Time of incubation	C^{14} in cellulose
		min.	*c.p.m.*
A	UDPG, 4 μmoles	0	168
B	" 4 "	120	175
C	" 4 " + 6 mg. cellodextrins	120	219
D	C^{14}-UDPG, 4.1 μmoles (30,500 c.p.m. per μmole) + 6 mg. cellodextrins	120	1004
E	UDP, 3.0 μmoles	120	225

cellulase, an increase in the amount of radioactive glucose is observed. Thus, in a typical large scale experiment, the glucose area on a paper chromatogram of the deionized supernatant fluid contained 345 c.p.m. before digestion with cellulase, presumably because of the breakdown of some UDPG during incubation. After cellulase digestion the glucose area on a paper chromatogram contained 2580 c.p.m. From the same

incubation mixture insoluble cellulose containing 4400 c.p.m. was isolated. The analysis of the supernatant fluid after deproteinization and deionization is somewhat complicated by the presence of a radioactive material with a mobility on paper similar to that of cellobiose. This compound is not digested with *M. verrucaria* cellulase, and after addition of cellobiose carrier does not crystallize with it. Its mobility on paper is different from that of maltose, sucrose, or melibiose, but similar to that of trehalose. Incubation of this compound with the enzyme in the presence or absence of UDPG or cellodextrins does not yield any radioactive cellulose in the usual assay system.

DISCUSSION

The data presented are consistent with a mechanism of cellulose synthesis by *A. xylinum* similar to the one previously described for chitin (2):

$$\text{UDPG} + (\text{glucose})_n \rightarrow \text{UDP} + (\text{glucose})_{n+1}$$

The reversibility of this reaction has not been demonstrated.

It is of interest that the enzyme synthesizing UDPG from uridine triphosphate and G-1-P (8) could be demonstrated in the supernatant fluid after centrifugation of the sonic extract at 140,000 × *g*.

Several attempts have been made to render soluble the enzyme catalyzing the synthesis of cellulose. Treatment with *n*-butanol or preparation of an acetone powder proved to be ineffective. Part of the activity could be obtained as a soluble system by extraction with 2 per cent digitonin in 0.05 M Tris-0.01 M $MgCl_2$-0.001 M EDTA, pH 7.5. Treatment with pancreatic lipase[5] (19) in the presence of digitonin and trypsin inhibitor gave rise to an active preparation which could not be sedimented by centrifugation at 140,000 × *g* for 1 hour. This enzyme, however, was extremely labile. It lost 30 per cent of the activity in 48 hours at −20° and did not withstand ammonium sulfate fractionation or dialysis.

The possibility that more than one enzyme is involved in cellulose synthesis in this system cannot at present be excluded. It has been suggested that the formation of cellulose fibrils may be caused by the action of extracellular enzymes (20, 21) which act on short chain cellodextrins produced by the cell, perhaps by transglycosidation. We have tested the addition of culture fluid, after removal of the cells and cellulose pellicle, to the assay system; no increase in the formation of insoluble cellulose was observed.

SUMMARY

A particulate enzyme preparation obtained from sonic extracts of *Acetobacter xylinum* is described which catalyzes the synthesis of cellulose

[5] We are grateful to Dr. R. K. Crane for this preparation.

from uridine diphosphoglucose in the presence of soluble cellodextrins. Some properties of the enzyme are discussed.

The author is indebted to Dr. D. H. Brown for many valuable suggestions. A gift of purified *Myrothecium verrucaria* cellulase from Dr. D. R. Whitaker is gratefully acknowledged.

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BIOSYNTHESIS OF COLLAGEN

III. THE DIRECT ACTION OF ASCORBIC ACID ON HYDROXYPROLINE AND COLLAGEN FORMATION IN SUBCUTANEOUS POLYVINYL SPONGE IMPLANTS IN GUINEA PIGS*

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(Received for publication, January 2, 1958)

Little is known about the biochemical processes involved in the formation of fibrous collagen. There is no question that ascorbic acid plays a prominent role in the early stages of collagen biosynthesis since the withdrawal of ascorbic acid from the diet of guinea pigs for a period merely long enough to deplete the animal results in the inability to form new collagen. Upon the administration of ascorbic acid the defect is promptly repaired (1). Earlier concepts (2, 3) that ascorbic acid acts as a gelating agent which transforms preexisting extracellular material into collagen may not be entirely accurate. Orekhovich and his coworkers (4, 5), Highberger, Gross, and Schmitt (6), and Gross (7) have identified soluble procollagenous compounds that are essentially similar to mature collagen in amino acid composition in the skin of growing young animals. Considerably diminished amounts were found in the skin of scorbutic animals. Gould and Woessner (1) have presented presumptive evidence that in the absence of ascorbic acid there is an apparent accumulation of a hydroxyproline-free precursor of collagen. In the absence of ascorbic acid the metabolic defect appears to be the inability to hydroxylate proline to hydroxyproline. Robertson and Schwartz (8) have also postulated such a precursor.

Data do not exist to clarify the role of ascorbic acid in such a hydroxylation mechanism or even to substantiate the concept of a direct local action for ascorbic acid in collagen formation or, for that matter, with respect to any biological effect of ascorbic acid. It is indeed possible that the failure of fiber formation in scurvy may be related to impaired adrenal hormone balance (9, 10), abnormal carbohydrate metabolism (11), or other general systemic alterations which result from ascorbic acid deficiency.

Quantitative studies of collagen formation by chick fibroblasts grown in roller tube cultures in synthetic as well as in complex media free from

* This investigation was supported by grant No. A-1270 from the Division of Research Grants and Fellowships, National Institutes of Health, Public Health Service.

The technical assistance of Lois B. Tait is gratefully acknowledged.

ascorbic acid have shown (12) that collagen formation can proceed in the absence of ascorbic acid and is not enhanced by ascorbic acid. Such results suggest that collagen formation in tissue culture is not dependent on ascorbic acid. However, the prompt cessation of rapid collagen formation, such as that encountered in wound healing and skin regeneration in guinea pigs concomitant with the depletion of ascorbic acid, is in marked contrast to the results observed in tissue cultures. It was desirable, therefore, to determine whether a direct local effect of ascorbic acid could be demonstrated in an active collagen-forming system. The technique of Boucek and Noble (13) which involves the subcutaneous implantation of polyvinyl (Ivalon) sponge which can serve as a scaffolding upon which connective tissue can be formed was suitably adapted for the study of collagen formation in normal, scorbutic, and ascorbic acid-treated guinea pigs. The method was found to be uniquely suited to the study of the local as well as the systemic effect of ascorbic acid on collagen biosynthesis.

Materials and Methods

Animals—Guinea pigs, weighing 250 to 300 gm. and averaging about 275 gm., were placed on a scorbutigenic diet (14) which resulted in first signs of scurvy within 13 to 15 days, and death generally occurred in 30 to 34 days. Normal control animals were fed this diet, supplemented daily with 10 or 20 mg. of L-ascorbic acid given orally by dropper. After the animals had been maintained on the scorbutigenic diet for 7 days to insure depletion of tissue and blood ascorbic acid, they were prepared as follows. The hair of the intrascapular region was removed, the skin was cleaned with 70 per cent alcohol, and an incision about 5 to 6 mm. long was made on each side of the mid-line. A polyvinyl sponge, prepared as described below, was inserted aseptically through each incision so that the sponge rested at a distance of 2 to 3 cm. from the incision. These incisions were then closed by means of a single suture.

Collection of Tissue—At various intervals after the implantation of the sponge, during which time the formation of connective tissue had proceeded as far as possible, the sponge was recovered by means of forceps through a small incision made directly above it. In most instances the removal was extremely simple, but when extensive collagen formation occurred, care was taken to avoid collecting any of the dense collagenous capsule that often surrounded the sponge.

The tissue-filled sponge, collected in this manner, was dried in acetone for 24 hours, minced finely, and reextracted with acetone for an additional 24 hours. The acetone-treated tissue was defatted with anhydrous ethyl ether, then transferred to a 13 × 100 mm. Pyrex tube and dried overnight at 108° in a vacuum oven.

Polyvinyl Sponge Implants—Polyvinyl Ivalon surgical sponge was used throughout. The sponge as obtained commercially was allowed to dry in air until it was stiff enough to slice on an electrically driven food-slicing machine. Sheets were cut about 3 mm. thick, and circles about 12 mm. in diameter were cut from these by means of a sharpened cork borer. Uniform circles which weighed between 19 and 20 mg. were selected, washed repeatedly to remove free formaldehyde, distributed in tubes of distilled water, and sterilized at a steam pressure of 15 pounds per sq. in. for 20 minutes. The wet sponge was implanted as described above.

Chemical Methods; Isolation of Collagen—The collagen of the dried sponge was converted to gelatin by adding 3 ml. of water to each sample tube and autoclaving for 3 hours at a steam pressure of 25 pounds per sq. in. The extract was transferred to a tared 13 × 100 mm. tube and the residue was reextracted with 2 ml. more of water by autoclaving as before. The resulting extracts were combined and evaporated to dryness in a current of air on a steam bath. The extract was dried to constant weight *in vacuo*.

Hydrolysis of Autoclaved Extract—The autoclave-extractable material was hydrolyzed in 6 N HCl in a sealed tube by heating at 150° for 3 hours. The hydrolysate was neutralized with the theoretical amount of NaOH and diluted as desired for analysis.

Hydroxyproline Determinations—These determinations were carried out by the method of Neuman and Logan (15) as modified by Martin and Axelrod (16). The hydroxyproline value multiplied by 7.46 may be taken as a measure of the apparent collagen content. This would include fibrous collagen along with precollagenous substances containing hydroxyproline. The results are generally expressed as micrograms of hydroxyproline in the total implant.

Preparation of Dehydroascorbic Acid—This compound was synthesized freshly before use by the method of Patterson (17), based upon the oxidation of ascorbic acid by benzoquinone in slightly acid solution. Ascorbic acid, dehydroascorbic acid, and diketogulonic acid were determined when necessary by the method of Roe *et al.* (18).

RESULTS AND DISCUSSION

Hydroxyproline Formation in Implanted Sponges in Normal and Scorbutic Guinea Pigs—Polyvinyl sponges (20 mg.) were implanted subcutaneously as described above in a group of animals placed on a scorbutigenic diet for 7 days. One set of guinea pigs was allowed to continue on the unsupplemented diet (Fig. 1, Curve A) throughout. A second set was replaced on the diet supplemented by 10 mg. of L-ascorbic acid daily (Curve B). After 12 additional days on the scorbutigenic diet, several animals which had received no L-ascorbic acid were placed on the supplemented diet and

maintained on it for several days (Curve C). The data indicate quite clearly that the formation of collagen in implanted sponges resembles to a striking degree that observed in skin regeneration (1).

The presence of hydroxyproline, in small but definite amounts, observed soon after implantation, appears to reflect a small infiltration of collagenous material into the sponge. Histological examination supports this view, since it reveals the presence of a few collagen fibers even though no vas-

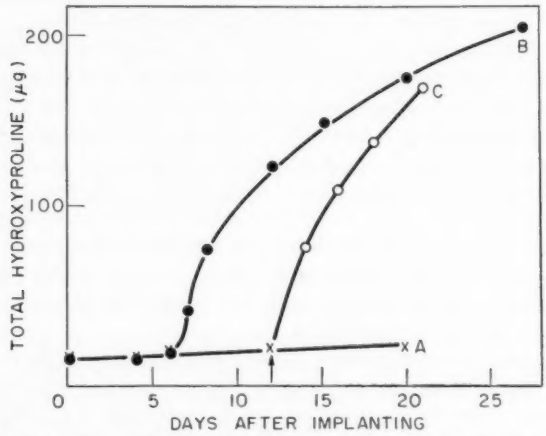

FIG. 1. Total hydroxyproline content of hydrolysates of autoclave-extracted material from polyvinyl (Ivalon) sponges implanted subcutaneously 7 days after withdrawal of ascorbic acid from the diet. The total hydroxyproline value multiplied by the factor 7.46 gives a measure of the apparent collagen. Curve A, animals maintained on a scorbutigenic diet throughout; Curve B, animals replaced on the diet supplemented by 10 mg. of ascorbic acid daily; Curve C, animals maintained for a further 12 days on the scorbutigenic diet and then given 10 mg. of ascorbic acid daily. The arrow indicates the point at which ascorbic acid was restored to Group C. Each point represents the average value for four to six animals.

cularization of the sponge has occurred. As in skin regeneration, little hydroxyproline is formed during the first 5 or 6 days and few fibers are noted histologically. From the 7th day onward, rapid hydroxyproline formation, accompanied by fiber formation, ensues. Upon the administration of L-ascorbic acid to the animals maintained on the scorbutigenic diet (Curve C) there is a prompt formation of hydroxyproline, which in 48 hours attains the level reached by the animals of Curve B in about 8 days. These data indicate that collagen formation in subcutaneous polyvinyl sponge implants follows a course analogous to that involved in collagen formation in actual skin regeneration (1).

Hydroxyproline Formation in Adult Guinea Pigs—Adult guinea pigs

weighing from 600 to 700 gm., which would not be expected to show any of the signs of scurvy until after they had been on a scorbutigenic diet for several months, were placed on such a diet for 7 days, after which time sponges were implanted in the usual way. The animals were maintained on the scorbutigenic diet for an additional 12 days, at which time the implants were removed and analyzed. The data indicate that there was essentially no hydroxyproline formed (21.0 ± 6.0 mg. per sponge) and support the

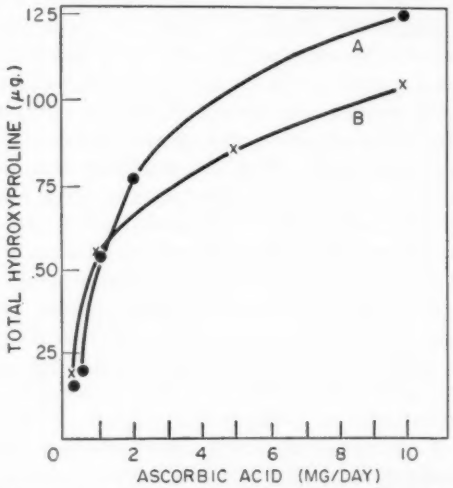

FIG. 2. Total hydroxyproline content of hydrolysates of autoclave-extracted material from polyvinyl (Ivalon) sponges implanted subcutaneously 7 days after withdrawal of ascorbic acid from the diet, after which time the animals were replaced on the scorbutigenic diet supplemented by varying amounts of orally administered ascorbic acid for a further 12 days, Curve A; or maintained on the scorbutigenic diet for an additional 12 days and then given the diet supplemented by varying amounts of ascorbic acid for a further 4 days, Curve B. Each point represents the average values for at least six animals.

findings in young (275 gm.) animals that after L-ascorbic acid depletion, which occurs within 5 to 7 days after withdrawal of dietary L-ascorbic acid, hydroxyproline biosynthesis ceases, even though there are no indications of scurvy.

Quantitative Relationship between Ascorbic Acid Administered and Hydroxyproline Formed—A set of experiments was carried out to determine the relative responses in hydroxyproline formation to varying amounts of orally administered L-ascorbic acid. One group of animals was fed varying amounts of L-ascorbic acid for 12 days after the implantation of the sponges (Fig. 2, Curve A). Another group was maintained on the scorbutigenic

diet for 12 days after implantation of the sponges and then given varying amounts of L-ascorbic acid for 4 days thereafter (Fig. 2, Curve B). It appears that relatively large amounts of orally administered L-ascorbic acid are needed for collagen formation in sponge implants as compared to the requirement for collagen formation in skin wounds. In skin wounds, the oral administration of 0.25 mg. of L-ascorbic acid leads to appreciable repair, and extensive repair occurs if 1.0 mg. is administered. A somewhat better response is noted in sponge implants when L-ascorbic acid is administered parenterally (Table I). The data point to the possibility that the implant may be organized in such a way that it presents a partial barrier to essential components of the active collagen-forming system. The direct relationship between the amount of ascorbic acid administered and the amount of hydroxyproline formed suggested that one of the components might be ascorbic acid itself. With the possibility that the implant may be organized to present a barrier to the loss of material from the collagen-forming site as well, experiments were carried out to determine whether ascorbic acid might not be involved directly in collagen formation.

Direct Interaction of L-Ascorbic Acid in Hydroxyproline and Collagen Biosynthesis—The prompt cessation of hydroxyproline formation in the sponges upon the withdrawal of L-ascorbic acid from the diet, along with the rather high orally administered dosage required for optimal activity, suggested that it might be possible to determine whether there is a local effect of L-ascorbic acid on collagen biosynthesis by injecting relatively small amounts of the compound directly into the sponge either throughout the period of collagen formation or during a short curative period.

Several groups of guinea pigs were prepared in the usual way and sponges were implanted bilaterally after a preliminary depletion period of 7 days, during which time the animals were on the scorbutigenic diet. Certain groups of animals were then injected, over a period of 12 days, with sodium ascorbate in 0.9 per cent sodium chloride solution into one sponge, and with 0.9 per cent sodium chloride solution into the second sponge. In this way each animal carried its own control. The daily amount administered was found to be most effective when injected in five equal doses uniformly spaced over an 8 hour period. The desired amount of any compound was always injected as the sodium salt, unless otherwise indicated, in a volume of 0.1 ml. Unless stated differently, the data refer to the results obtained with divided doses and the amounts indicated refer to the total dose. Other groups of animals were continued on the scorbutigenic diet for 12 days after the sponges had been implanted. At this point varying amounts of sodium L-ascorbate in saline were injected directly into one sponge in divided doses, and the control sponge was similarly injected with saline over a period of 4 days, at which time the sponges were removed for analysis.

There is a clear indication (Fig. 3) of a pronounced local effect of L-ascorbic acid on hydroxyproline formation. Curve A represents the influence of

TABLE I

Summary of Data Demonstrating Direct Action of L-Ascorbic Acid in Hydroxyproline Synthesis in Implanted Polyvinyl Sponges in Ascorbic Acid-Depleted Guinea Pigs

Sodium L-ascorbate administered	No. of animals	Hydroxyproline (total)*		
		Ascorbate-injected sponge	Saline-injected sponge	Subcutaneous injection
		γ	γ	γ
0 mg. 12 days	3 (600 gm.)		21.0 ± 6.0	
	22 (275 ")		13.1 ± 3.5	
0.1 mg.				
For 12 days (single daily dose)	21	45.7 ± 7.5	23.3 ± 5.4	
For 12 days (divided daily dose)	4	48.0 ± 13	9.0 ± 1.2	
0.0 mg. for 12 days followed by 0.1 mg. for 4 days	9	44.0 ± 7.9	21.9 ± 7.2	26.9 ± 6.0
0.25 mg.				
For 12 days	3	150 ± 20	54.0 ± 22	
0.0 mg. for 12 days followed by 0.25 mg. for 4 days	10	61.8 ± 15.9	12.5 ± 3.1	
0.50 mg.				
For 12 days	14	111.4 ± 23.5	43.6 ± 15.3	44.8 ± 5.4
0.0 mg. for 12 days followed by 0.5 mg. for 4 days (single dose)	3	69.3 ± 17	29.3 ± 3.7	33.3 ± 3.3
As above in divided dose	10	126.1 ± 22.7	33.8 ± 9.1	36.5 ± 0.5
1.0 mg.				
0.0 mg. for 12 days followed by 1.0 mg. for 4 days	5	138 ± 40.5	56.0 ± 4.6	

* All values are expressed as the total amount of hydroxyproline per sponge and the data are presented as the mean ± its standard deviation. The hydroxyproline value multiplied by 7.46 may be taken as a measure of the apparent collagen. Each animal carried both a treated and a control sponge. In every case the ascorbic acid-treated sponges contained greater amounts of hydroxyproline.

dosage on the amount of hydroxyproline formed in the ascorbic acid-treated sponge, and Curve B the amount of hydroxyproline formed in the saline-treated control sponges. It is evident that, when 1.0 mg. of sodium L-

ascorbate is introduced into one sponge, enough reaches the control sponge through the circulation to influence the production of hydroxyproline in it. The amount of hydroxyproline formed suggests that the equivalent of 0.1 mg. per day reaches the control sponge under these conditions. This was further demonstrated when 1.0 mg. of sodium ascorbate was

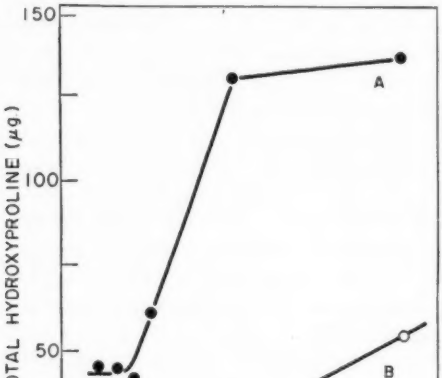

FIG. 3. Total hydroxyproline content of hydrolysates of autoclave-extracted material from polyvinyl sponges implanted bilaterally under the skin of guinea pigs 7 days after withdrawal of ascorbic acid, at which time the animals were continued on the scorbutigenic diet for 12 days and thereafter injected for 4 days with varying amounts of sodium ascorbate in 0.9 per cent sodium chloride solution into one sponge, Curve A; or with 0.9 per cent sodium chloride solution alone, Curve B. The total amounts indicated on the abscissa were administered daily over an 8 hour period in five equal doses. Each point represents the average value for a group of five to ten animals. Each pair of points at any dose represents the same group of animals.

injected subcutaneously in a control set of animals. Under these conditions, amounts of hydroxyproline comparable to those found in the control sponges of animals receiving 1 mg. in the test sponge were formed. It would appear that the injection of 0.5 mg. of sodium L-ascorbate daily into the site of collagen formation is comparable to the daily oral administration of about 10 mg. of L-ascorbic acid.

Even when as little as 0.1 mg. of sodium L-ascorbate was injected, there

was a significantly greater synthesis of hydroxyproline than that in the control sponges in the same animals (Table I). The difference between treated and control sponges was noted in practically every guinea pig studied, even at these low levels, which are far below the minimal critical levels (about 0.25 to 0.30 mg. of sodium ascorbate) required for the maintenance of histological tooth structure (19) or bone and serum phosphatase levels (20). Histological examination of sponges injected with 0.25 to 1.0 mg. of sodium ascorbate daily during a 12 day preventive period, or after 4 days of curative treatment, reveals the abundant presence of collagen fibers in the treated sponges, with practically no fibers or minimal fiber formation in the control sponges.

If amounts greater than 1.0 mg. of sodium ascorbate daily are injected into implanted sponges, large quantities of the compound enter the circulation so that the animal produces large amounts of hydroxyproline and collagen in both sponges (Table I). However, in a series of guinea pigs in which 5.0 mg. of sodium L-ascorbate were injected into one sponge and saline into the other, it was found that in each case the sponge injected with sodium L-ascorbate contained the greater amount of hydroxyproline. The average was 199 γ of hydroxyproline in the treated sponge as compared to 144 γ in the control sponge. When 5.0 mg. of sodium L-ascorbate were injected subcutaneously as a control in a similar series of animals, the average content of hydroxyproline was found to be 142 γ. In addition to demonstrating what appears to be an enhanced effect on collagen formation when L-ascorbic acid is introduced directly into the site of fiber formation, this result also suggests that high levels of ascorbic acid do not inhibit the mechanism.

Additive Hydroxyproline Formation Resulting from Simultaneous Local and Oral L-Ascorbic Acid Administration—A group of guinea pigs was prepared in the usual manner, sponges were implanted, and the animals were maintained on the scorbutigenic diet for an additional 12 days. They were then fed 10 mg. of L-ascorbic acid daily for the next 4 days, and during this same 4 day period 0.5 mg. of sodium L-ascorbate was injected (in divided doses) daily into one sponge while the control sponge was injected with saline. It was found that the control sponges averaged 175 $\pm$ 8.5 γ of hydroxyproline, whereas the ascorbic acid-injected sponges in these same animals averaged 240 $\pm$ 24 γ of hydroxyproline. The oral administration of 10 mg. of L-ascorbic acid under these conditions could be expected to produce 105 γ of hydroxyproline in each sponge (Fig. 2) in an animal. The injection of 0.5 mg. of sodium L-ascorbate per day into the sponge would be expected to result in 138 γ of hydroxyproline in the treated sponge and about 36 γ in the control sponge. If the effects are additive, the anticipated values should be 243 γ of hydroxyproline in the

treated sponge as compared with 141 γ in the control. From the observed results, it may be concluded that there is an added local effect when L-ascorbic acid is injected into a regenerating site in an animal that is at the same time receiving ascorbic acid orally.

Specificity of L-Ascorbic Acid in Hydroxyproline Biosynthesis—Zilva (21) has suggested that the ineffectiveness of certain of the analogues of ascorbic acid is due to the inability of the tissues to retain them. Sealock *et al.* (22) have demonstrated that L-ascorbic acid can stimulate tyrosine oxidation by scorbutic liver slices but that D-glucoascorbic acid and reductone are equally effective. The present system involving what appears to be a direct effect of L-ascorbic acid on the biosynthetic mechanism seems well suited to determine whether L-ascorbic acid is specific in collagen biosynthesis.

Several series of animals were prepared as usual, sponges were implanted, and the animals maintained on the scorbutigenic diet for a further 12 days. During the next 4 days implants in these animals were injected with the sodium salts of either D-glucoascorbic acid, D-isoascorbic acid, or dihydroxymaleic acid. The results (Table II) indicate that neither the dihydroxymaleate, whose enediol structure has been associated with pronounced effects on the intercellular mucopolysaccharides and ground substance (23), nor D-glucoascorbate, which has no effect as an antiscorbutic substance, has any significant local effects on hydroxyproline formation. Glucoascorbate, whose antagonistic action in ascorbic acid systems has been controversial (24, 25), was found to show no serious biochemical antagonism when injected into sponges of animals receiving L-ascorbic acid by mouth. Similarly, when D-isoascorbic acid was injected into sponges in animals which received 10 mg. of L-ascorbic acid by mouth, no significant additive effect was observed.

These results suggest that the specific structure of L-ascorbic acid is required for hydroxyproline biosynthesis, since neither the compounds containing the enediol configuration nor ascorbic acid analogues with comparable reducing activity are effective.

Dehydroascorbic Acid—This compound has been shown to be antiscorbutic (20), perhaps because of its reduction to L-ascorbic acid. A series of animals similar to that described above was studied. One sponge was injected with freshly prepared dehydroascorbic acid (17) or with the methanol complex of dehydroascorbic acid. The data (Table II) indicate clearly that, as in the over-all scorbutic process (20), dehydroascorbic acid is almost as effective as L-ascorbic acid in the direct stimulation of hydroxyproline formation. It is not yet possible to state whether dehydroascorbic acid is reduced before it becomes active, nor is it yet clear whether L-ascorbic acid, dehydroascorbic acid, or perhaps an intermediate form is the active material.

Possible Multiple Pathways for Collagen Biosynthesis—The results of these experiments which indicate a local action for ascorbic acid in collagen biosynthesis, when compared with the results obtained from tissue culture experiments where no such direct effect is apparent, suggest that more than one pathway for collagen formation may exist. The extremely slow turnover of body collagen, as compared with such rapid synthesis as that encountered in tissue repair and in an experimental system like the one employed in this study, also favors such a possibility. It is not improbable that the mechanism for rapid collagen formation requires ascorbic acid as a component while in the slow normal turnover the mechanism involved is independent of ascorbic acid. It is also possible that the same general mechanism may be involved but that ascorbic acid can serve to release an inhibitory mechanism which operates under normal conditions, resulting in rapid biosynthesis of collagen.

TABLE II

Influence of Certain Components Showing Structural Similarity to L-Ascorbic Acid upon Local Stimulation of Hydroxyproline Formation in Implanted Polyvinyl Sponges in Ascorbic Acid-Depleted Guinea Pigs

Treatment		No. of animals	Hydroxyproline (total)*	
			Ascorbate-injected sponge	Saline-injected sponge
			γ	γ
L-Dehydroascorbic acid	0.5 mg. freshly prepared	3	110 ± 12.6	14.0 ± 2.3
	0.7 " as methanol complex	5	116 ± 32	24.6 ± 2.8
Dihydroxymaleic acid	0.37 mg. per day into sponge	5	13.6 ± 2.2	14.2 ± 4.3
D-Glucoascorbic acid	0.5 mg. per day into sponge	9	20.5 ± 7.6	12.5 ± 2.6
D-Glucoascorbic acid	1.0 mg. per day into sponge + 10 mg. L-ascorbic acid orally	5	130 ± 30.8	120 ± 20.7
D-Isoascorbic acid	0.5 mg. per day into sponge + 10 mg. L-ascorbic acid orally	5	128 ± 12.8	104 ± 20
L-Ascorbic acid	0.5 mg. per day into sponge + 10 mg. orally	5	240 ± 24	175 ± 8.5

* All animals were placed on a scorbutigenic diet for 7 days before sponges were implanted, and were maintained on the diet for a further 12 days. At this point they were treated for 4 days as indicated. Oral doses were given once daily and the injected doses were divided. All acids were administered as the sodium salts except dehydroascorbic acid, which is unstable. Each animal carried both a treated and a control sponge.

SUMMARY

Collagen biosynthesis as measured by hydroxyproline synthesis in subcutaneously implanted polyvinyl sponges has been studied in normal, scorbutic, and ascorbic acid-treated guinea pigs. It has been found that hydroxyproline formation in such a system under various conditions is analogous to hydroxyproline formation in granulation tissue.

A direct, specific effect for ascorbic acid in collagen biosynthesis *in vivo* has been demonstrated.

The administration of relatively small doses of sodium L-ascorbate into implanted sponges in previously ascorbic acid-depleted guinea pigs resulted in rapid hydroxyproline synthesis in the treated sponge with little or no synthesis in the control sponge in the same animal. When ascorbic acid was administered by mouth at the same time that it was introduced directly into the site of collagen formation, an additive effect was observed.

The direct action of ascorbic acid appears to be specific for substances of known antiscorbutic activity since dihydroxymaleic acid, glucoascorbic acid, and isoascorbic acid are inactive, whereas L-ascorbic acid and L-dehydroascorbic acid are active.

It appears that rapid collagen biosynthesis such as that involved in tissue repair may be directly mediated by ascorbic acid, in contradistinction to collagen formation in tissue culture which appears to be independent of ascorbic acid, suggesting the possibility of more than a single biosynthetic pathway.

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FURTHER STUDIES ON THE INTRODUCTION OF PYRIMIDINES INTO DEOXYRIBONUCLEIC ACIDS OF ESCHERICHIA COLI*

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(Received for publication, January 15, 1958)

Previous studies (3) have been concerned with the introduction of halogenated pyrimidines into the deoxyribonucleic acids (DNA) of *Escherichia coli*. These studies have now been extended to other pyrimidines, other conditions, and other strains in an attempt to make the phenomenon more general and to apply it eventually to higher organisms.

EXPERIMENTAL

Materials, bacterial cultures, and strains were the same as those described previously (3). In addition, the following substances and strains were used: thymidine and 5-nitrouracil (Nutritional Biochemicals Corporation, Cleveland, Ohio); 5-bromouracil deoxyriboside and 5-methylcytosine (California Foundation for Biochemical Research, Los Angeles); 5-bromouracil riboside (synthesized by the method of Fukuhara and Visser (4)); 6-methylaminopurine (kindly supplied by Dr. Gertrude Elion, Wellcome Research Laboratories, Tuckahoe, New York); Aminopterin (Lederle Laboratory Division, American Cyanamid Company, New York); a mutant of strain 15 (Roepke) that requires phenylalanine (ATCC 9723f); another mutant of strain 15, requiring uracil, cytosine, or isocytosine (ATCC 9723g); a mutant of strain W that requires a purine (5); and a tryptophan-requiring mutant B3/1 derived from a thymine-requiring mutant B3 (Brenner) of strain B, kindly supplied by Dr. Ernst Freese, Harvard University.

Isolation of highly polymerized DNA (3) and the determination of growth inhibition (6) were performed as described previously.

The determination of viscosity, heat stability, and ultraviolet absorption spectra of a DNA preparation containing 5-bromouracil was also performed as described by Zamenhof *et al.* (7).

* This investigation was supported by research grant No. C-1760 from the National Institutes of Health, Public Health Service, grant No. E-52 from the American Cancer Society, and grant No. G-4337 from the National Science Foundation. Abstracts of certain parts of this investigation have been presented previously (1, 2).

Analytical Procedures

The analytical procedures were those already described, except that for the estimation of ribonucleic acid (RNA) the more accurate micromodification of the phloroglucinol method (8) was used instead of the orcinol method. The following procedures were also used.

Chromatographic Separation of 5-Nitrouracil—DNA was isolated, freed from RNA by alkali treatment followed by dialysis, and hydrolyzed with $HClO_4$ as described (3). The hydrolysate was deposited in a row of at least twenty-five 0.001 ml. spots on the starting line of each of four Whatman No. 50 filter paper strips. After neutralization in gaseous ammonia, the strips were subjected to chromatography, with water as the solvent (9). After 8 hours, the pyrimidine band was clearly separated from the purine band. The pyrimidine band was cut out and eluted in water; the eluate was then concentrated and subjected to chromatography for 48 hours in butanol-NH_3, as described in an earlier paper (3). In this system, 5-nitrouracil migrates at a slower rate than any other natural pyrimidine (R_F 5-nitrouracil:R_F cytosine = 0.4).

Chromatographic Separation of 5-Methylcytosine and of 6-Methylaminopurine—This procedure (10) was performed essentially as that described for 5-nitrouracil, except that in the first step (63 hours) an isopropanol-2 N HCl mixture (11) rather than water was used as a solvent. The confluent bands, corresponding to the levels of cytosine, 5-methylcytosine, and 6-methylaminopurine of the control lane, were cut out together and eluted in water; the eluate was then concentrated and subjected to chromatography for 96 hours in butanol-NH_3, as described for 5-nitrouracil. In this system, the R_F cytosine:R_F 5-methylcytosine = 0.82; R_F cytosine:R_F 6-methylaminopurine = 0.61.

Estimation of Thymine Content—The DNA hydrolysate (see above) was deposited as single 0.001 ml. spots on Whatman No. 50 filter paper strips, subjected directly to chromatography for 68 hours in butanol-NH_3, and the molar ratios of adenine to cytosine to thymine were determined as described by Zamenhof *et al.* (3). No attempt was made to isolate guanine since it is partially destroyed during the hydrolysis in $HClO_4$.

RESULTS AND DISCUSSION

Introduction of 5-Bromouracil into DNA of Strains Not Requiring Thymine—In a previous communication (3) it was reported that, whereas the thymine-requiring strain I, grown on enriched broth containing 100 γ of 5-bromouracil per ml., replaces 48 mole per cent of thymine by 5-bromouracil, the wild strain B (not requiring thymine) grown under the same conditions replaces only 4.4 mole per cent. First, it was of interest to ascertain that the high amount of thymine replacement is not a charac-

teristic of the parent Roepke strain (12) from which strain I originated. For this purpose, an unrelated (phenylalanine-requiring) mutant of the parent strain (ATCC 9723f) was grown under similar conditions, except that the 5-bromouracil content was raised to 200 γ per ml. The low thymine replacement (7.5 mole per cent, Table I) indicates that the replacement is correlated with a "disturbance" in DNA synthesis rather than with the origin of the strain. This is also clearly indicated by the finding that in a thymine-requiring mutant of strain B the replacement was eleven times higher (48 mole per cent, Table I) than in the wild strain B (4.4

TABLE I

Thymine Replacement by 5-Bromouracil

Strain No.	Growth requirement	5-Bromouracil	Aminopterin	Thymine replacement
		γ per ml.	*γ per ml.*	*mole per cent*
I	Thymine	100		48*
II	None	100		14†
9723f (ATCC)	Phenylalanine	200		7.5
9723g (ATCC)	Uracil	200		18
B	None	100		4.4*
"	"	100	250	13.5
"	"	2000		17.0
"	"	6000		0
B3/1	Thymine and tryptophan	100		48
W‡	Purine	2000		13.0

* Zamenhof *et al.* (3).
† Zamenhof and Griboff (13).
‡ Davis (5).

mole per cent) grown under the same conditions. It is not unlikely that any species can be made to replace thymine by 5-bromouracil if the species, or its mutant, requires thymine for growth.

A mutant which requires uracil may be considered to be a partial "thymine requirer," since in this strain uracil is likely to be a precursor of thymidylic acid. One such strain (ATCC 9723g) is not inhibited by 100 γ of 5-bromouracil, 2-thiothymine, 5-bromouracil riboside, or 5-bromouracil deoxyriboside per ml. of basal broth (6), thus indicating that thymine or its nucleosides may not be the intermediates. When this strain was grown under similar conditions as the phenylalanine requirer, 5-bromouracil replaced much more thymine (18 mole per cent, Table I), suggesting the correlation mentioned above. This result may have a

practical significance for studies of the introduction of thymine analogues into DNA: thymine-requiring mutants or thymine-requiring wild strains are rare, whereas those requiring uracil are comparatively frequent.

The "back-mutant" of strain I to strain II (not requiring thymine) replaces 14 mole per cent of thymine (13) on 100 γ of 5-bromouracil per ml. (Table I); this replacement, higher than in wild strain B, suggests that in strain II the pathway of DNA synthesis did not return to "normal." Indeed, this strain is eventually inhibited by 2000 γ of 5-bromouracil per ml. of basal broth, whereas the wild strain B is not (6). We have previously reported that strain I is inhibited by 0.5 γ of 5-bromouracil deoxyriboside per ml. of basal broth (13). It has now been found that under these conditions strain II is also inhibited, whereas wild strain B as well as the original Roepke strain 15 and its mutants (phenylalanine-requiring or uracil-requiring) is not inhibited even by 200 γ of this nucleoside per ml. It is likely that the pathway of DNA synthesis in this strain does not include thymine (2, 13) but does include thymidine as an intermediate. Such a pathway may account for higher thymine replacement than in the wild strain.

The wild strain B replaces thymine by 5-bromouracil (14) if it becomes a thymine requirer by growing in the presence of sulfanilamide (see also Cohen and Barner (15)). It has now been found that a similar result can be obtained by blocking methylation by means of Aminopterin. When strain B was grown on enriched broth containing 100 γ of 5-bromouracil and 250 γ of Aminopterin per ml., the thymine replacement was much higher (13.5 mole per cent, Table I) than without Aminopterin (4.4 mole per cent).

It occurred to us that the wild strain might also be induced to choose an alternative pathway, one which utilizes exogenous pyrimidine, when such a pyrimidine is present in very high concentration. Indeed, when strain B was grown in enriched broth containing 2000 γ of 5-bromouracil, the thymine replacement increased to 17 mole per cent (Table I). Similar results were obtained with strain W. This method may be useful for the introduction of 5-bromouracil into higher organisms which do not require thymine.

When the concentration of 5-bromouracil was increased to 6000 γ per ml., the cells were too much inhibited and the thymine was not replaced (Table I) in accordance with previous findings (6).

6-Methylaminopurine in DNA Containing 5-Bromouracil—Dunn and Smith (10) reported an increase (up to 30 mole per cent) in the 6-methylaminopurine content in DNA of cells grown under the conditions of thymine requirement or of inhibition of thymine utilization; this inhibition was achieved by using inhibitory thymine analogues (2-thiothymine,

5-aminouracil) which are not introduced into DNA (3, 10). It seemed of interest to determine whether such an increase also occurs when the analogue is introduced in place of thymine into DNA. Recently Dunn and Smith (16) found a small increase in the 6-methylaminopurine content during the synthesis of DNA on basal medium containing 5-bromouracil. The conditions used by these authors were different from those in the present work (introduction of 5-bromouracil without apparent DNA synthesis (3)). It was now found that in enriched broth, without or with 100 γ of 5-bromouracil per ml., the 6-methylaminopurine content of DNA in strain I was 1.6 mole per cent (of thymine) without and 2.7 mole per cent with 5-bromouracil. The difference is not considered significant. On the other hand, the 5-bromouracil content is none without, and 48 mole per cent with 5-bromouracil. These results indicate that when the organism can introduce an analogue into DNA (under the conditions of impaired thymine utilization) it does so in preference to introducing more 6-methylaminopurine, at least under the condition of the experiment. This finding is of importance for the study of the biological effects of the introduction of 5-bromouracil into DNA (to be discussed elsewhere). It indicates that such effects are unlikely to be caused by the change in 6-methylaminopurine content in DNA, since practically none occurs in these conditions.

Attempts to Introduce 5-Nitrouracil, 5-Methylcytosine, and Excess Thymine into DNA—Since an increase in the concentration of 5-bromouracil, up to 2 mg. per ml., was effective in introducing this analogue into DNA, a similar attempt was made to introduce 5-nitrouracil. Whereas there was no growth inhibition in basal broth containing 1 γ of thymine and 100 γ of 5-nitrouracil per ml. (13), we now find growth inhibition at 2 mg. of 5-nitrouracil per ml. This inhibition can be reversed by thymine or thymidine (40 γ per ml.). When strain I was grown in the presence of 2 mg. of 5-nitrouracil per ml. of enriched broth (no inhibition), the presence of this analogue in DNA could not be demonstrated. This finding again indicates the importance of the right size and charge of substituent in position 5 of uracil (3, 16).

5-Methylcytosine, a natural constituent of DNA of higher plants and animals, has never been found in bacterial DNA. In *E. coli*, this may be because of the presence of a deaminase which converts 5-methylcytosine deoxyriboside into thymidine (Cohen and Barner (17)). Nevertheless, an attempt was made to introduce 5-methylcytosine into DNA by using a strain (ATCC 9723g) which requires uracil or cytosine (1 γ per ml.) for growth, at relatively high concentrations of 5-methylcytosine (100 γ and 2 mg. per ml. of enriched broth). The cells were grown and the DNA was isolated and analyzed as described above. The presence of 5-methylcytosine in the DNA of these cells could not be demonstrated.

The thymine content in the DNA of a thymine-requiring strain of *E. coli*, grown on media containing 10 γ of thymine per ml., was found to be essentially equal to that of adenine and cytosine (18). It was of interest to investigate whether the thymine content could be raised by growing the cells in the presence of high concentrations of thymine or thymidine. When strain I was grown in enriched broth containing 6 mg. of added thymine per ml., the molar base ratios in DNA (averages of three experiments each: thymine:cytosine 1.11; adenine:thymine 1.05) were not significantly different from those when thymine was not added (thymine:cytosine 1.04; adenine:thymine 1.04). This strain requires four times less thymidine than thymine (on a molar basis) (13). It is now found that this strain is inhibited to the extent of 50 per cent by 1 mg. of thymidine per ml. of basal broth. In enriched broth containing 2 to 4 mg. of thymidine per ml., the cells become enlarged and distorted and the viability is only 0.4 to 3 per cent at 8 hours. The DNA of the cells grown under these conditions again showed no significant deviations in molar base ratios (thymine: cytosine 1.10; adenine:thymine 0.93).

It is of interest that Dunn and Smith (10) were also unable to increase the 6-methylaminopurine content in DNA by growing the cells in the presence of this naturally occurring pyrimidine. It is likely that in general the proportions of the naturally occurring bases in DNA cannot be changed by the presence of these bases in the medium even in relatively high concentrations.

Properties of DNA Containing 5-Bromouracil—5-Bromouracil has a different ultraviolet absorption maximum (276 mμ at pH 7) from thymine (264.5 mμ at pH 7). It was therefore conceivable that the absorption maximum of DNA, in which 32 mole per cent of thymine is replaced by 5-bromouracil, would be higher than that of normal DNA. However, the absorption maximum of such DNA containing 32 mole per cent of 5-bromouracil was found to be 259 to 260 mμ, which is not different from the normal.

The specific viscosity of the solution of this 5-bromouracil containing DNA (1800 γ of DNA per ml. of "standard buffer" (7)) was $\eta_{sp} = 12.1$ at 23°, which is practically the same as that for DNA of calf thymus ($\eta_{sp} = 11.7$) under identical conditions (7).

It is conceivable that the hydrogen bonds in the DNA containing 5-bromouracil are under more strain than those in normal DNA. It was recently reported that the cells of *E. coli* containing 5-bromouracil in their DNA are up to 2000 times more sensitive to ultraviolet irradiation (on a survival basis) than are the normal cells (19). This effect was correlated with the amount of thymine replacement by 5-bromouracil in DNA and not with the extent of inhibition by 5-bromouracil. It seemed of interest to investigate whether such DNA has a lowered resistance to heat which

occurs when the DNA molecules have undergone injury (7). The solution of DNA containing 5-bromouracil described above was heated to 76° for 1 hour, cooled to 23°, and the viscosity measured again (7). No loss of viscosity could be detected, which indicates that the resistance to heat was not impaired.

SUMMARY

The study of the introduction of 5-bromouracil into deoxyribonucleic acid (DNA) of *Escherichia coli* has been extended to various pyrimidines, various conditions, and several strains. The extent of such an introduction (thymine replacement) was correlated with the "disturbances" in DNA synthesis. Such disturbances occur naturally in several mutants (thymine requirers, their "back-mutants," uracil requirers) or can be produced artificially in wild strains by Aminopterin or by high concentrations of 5-bromouracil. This last method was, however, ineffective in introducing into DNA any 5-nitrouracil or 5-methylcytosine, or more thymine than exists normally. The ultraviolet absorption spectrum, the viscosity, and the heat stability of DNA containing 5-bromouracil have been investigated. These properties could not be distinguished from those of normal DNA.

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THE INHIBITION OF MITOCHONDRIAL RESPIRATION BY 1,10-PHENANTHROLINE AND 2,2′-BIPYRIDINE AND THE POSSIBLE RELATIONSHIP TO OXIDATIVE PHOSPHORYLATION*

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(Received for publication, January 6, 1958)

Chelating agents such as 1,10-phenanthroline and 2,2′-bipyridine have strong affinities for certain metal ions important in biological oxidation (1). There is little information on the effects of these substances on the tricarboxylic acid cycle. Glutamate oxidation by brain mitochondria was markedly inhibited by 1 mM 1,10-P[1] while 2,2′-B inhibited it only slightly (2). Citrate oxidation by liver mitochondria was inhibited 37 and 86 per cent, respectively, by 2,2′-B at 0.33 mM and 1 mM (3). No single cycle enzyme system has been found to be particularly sensitive; for example, 1 mM 1,10-P inhibited pig heart aconitase about 50 per cent (4), DPNH cytochrome *c* reductase 20 per cent (5), and succinic dehydrogenase 23 to 33 per cent (6). 2,2′-B was generally a weaker inhibitor of these enzymes. Nevertheless, it has been observed in this laboratory that pyruvate oxidation by rat heart mitochondria was quite potently inhibited by 1,10-P, and it was evident that some reaction in the cycle was more sensitive to this substance than any of the enzymes previously studied. The present investigation is concerned with the inhibition patterns produced by 1,10-P and 2,2′-B in these heart mitochondria and the localization of the site of action.

Methods

The preparation of the rat heart mitochondria suspension and the manometric measurement of oxygen uptake were according to Montgomery and Webb (7). The reaction medium, unless specified otherwise, contained the following: KCl (121 mM), potassium phosphate buffer (20 mM, pH 6.8), cytochrome *c* (0.01 mM), $MgCl_2$ (5 mM), AMP (1 mM), and ATP (0.5 mM). Rat liver mitochondria were prepared by the isotonic sucrose method (8). The α-ketoglutaric dehydrogenase was prepared from pig heart and assayed

* This investigation was supported by a grant from the Life Insurance Medical Research Fund and aided by facilities supplied by the Allan Hancock Foundation.

[1] The abbreviations used are as follows: 1,10-P, 1,10-phenanthroline; 2,2′-B, 2,2′-bipyridine; DPN, diphosphopyridine nucleotide; TTC, 2,3,5-triphenyltetrazolium chloride; DNP, 2,4-dinitrophenol.

spectrophotometrically (9). Malic dehydrogenase was also prepared from pig heart (10) and assayed by the method of Mehler *et al.* (11). DPNH cytochrome *c* reductase was prepared from pig heart (12), and its activity was determined by cytochrome *c* reduction. The cytochrome oxidase activity of the mitochondrial suspensions was determined manometrically with *p*-phenylenediamine as substrate. The ability of the mitochondria to oxidize added DPNH was determined both manometrically and spectrophotometrically.

The reduction of TTC was used as a measure of the rate of oxidation of certain cycle substrates according to a modification of the method of Kun and Abood (13). The following additions were made to each tube: 1 ml. of mitochondrial suspension, 1 mg. of TTC in 1 ml. of 0.1 M phosphate buffer at pH 7.2, 0.4 ml. of 50 mM substrate, 2 mg. of DPN, and 0.1 ml. of 0.01 mM methylene blue. When chelating agents were used, 0.3 ml. of a solution (10 times the final concentration) in 0.9 per cent KCl was added. The final volume was brought to 3 ml. with 0.9 per cent KCl in all cases. The incubation time varied from 60 to 120 minutes at 30°. Formazan was extracted with 5 ml. of cold acetone, the mixture was centrifuged, the clear acetone solution was transferred into cuvettes, and the concentration was determined spectrophotometrically at 485 mμ. Precaution was taken so that the tubes were not exposed to direct light during the incubation or extraction. For P:O determinations, mitochondrial oxidation of α-ketoglutarate was measured under the conditions previously described for a period of 20 minutes; the flask contents were deproteinized by the addition of cold 10 per cent trichloroacetic acid and analyzed for inorganic phosphate by the method of Lowry and Lopez (14).

Results

Inhibition of Oxidations in Heart Mitochondria—The average results of three experiments are shown in Table I. Marked inhibition of the oxidation of malate, pyruvate + malate, and α-ketoglutarate was observed with 0.01 to 0.03 mM 1,10-P, whereas the oxidation of citrate, isocitrate, and succinate was much less sensitive; the concentration of 2,2′-B required for maximal inhibition was approximately 10 times as great. A survey of a wide range of concentrations of 1,10-P and 2,2′-B on mitochondrial α-ketoglutarate oxidation gave Curves A and B in Fig. 1. A sufficient increase in concentration reduced the inhibition and occasionally produced stimulation; this was also found to be true for the oxidation of malate and pyruvate + malate. It was impossible to determine the effects of these agents on pyruvate oxidation alone, because rat heart mitochondria do not oxidize pyruvate in the absence of a cycle intermediate to provide oxalacetate (7). The three different peaks in the 2,2′-B concentration-inhibition curve were consistently obtained in each of the three experiments.

Action of Chelating Agents on Isolated Enzymes—Various enzymes involved in electron transport during oxidation of α-ketoglutarate and

TABLE I

Effects of Chelating Agents on Mitochondrial Oxidation of Various Substrates

Substrate	Per cent change					
	1,10-Phenanthroline			2,2′-Bipyridine		
	0.01 mM	0.03 mM	0.10 mM	0.30 mM	1.0 mM	5.0 mM
α-Ketoglutarate	−92.4	−100.0	−74.7	−79.3	−22.8	−9.0
Pyruvate + malate	−96.8	−92.7	−82.4	−87.9	−56.8	−14.5
Malate	−82.2	−80.8	−77.1	−66.8	−24.4	+3.3
Citrate	−32.9	−55.9	−56.9	−37.0	−26.3	−1.1
Isocitrate	+5.6	−43.5	−41.8	−20.7	−16.9	−22.3
Succinate	−14.5	−29.1	+3.7	−32.7	−1.2	−8.5

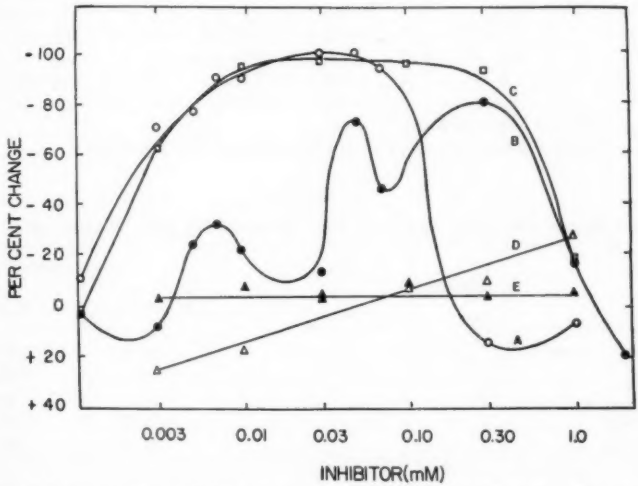

FIG. 1. Concentration-inhibition curves for 1,10-P and 2,2′-B. Curves A and B, inhibition of mitochondrial oxidation of α-ketoglutarate by 1,10-P and 2,2′-B, respectively; Curve C, inhibition of mitochondrial reduction of TTC by 1,10-P with α-ketoglutarate as the substrate; Curves D and E, inhibition of DPNH cytochrome *c* reductase activity by 1,10-P and 2,2′-B, respectively.

malate were tested in order to determine the site of inhibition. The results are summarized in Table II. Neither 1,10-P nor 2,2′-B at concentrations markedly inhibitory in mitochondrial oxidations produced significant inhibition of the α-ketoglutaric dehydrogenase, malic dehydrogenase,

DPNH cytochrome *c* reductase, or cytochrome oxidase, even after preincubation of the enzymes with the chelating agents. The inhibitions of DPNH cytochrome *c* reductase (Fig. 1, Curves D and E) are similar to those

TABLE II

Effects of Chelating Agents on Various Components of Electron Transport System

Agent	Concentration	Per cent change						
		α-Ketoglutaric dehydrogenase		Malic dehydrogenase		DPNH cytochrome *c* reductase		Cytochrome oxidase
		A*	B†	A	B	A	B	A
	mM							
1,10-P	0.01	−1.3	−15.0	+43.6	+152.0	−5.3	+0.8	+1.3
"	0.03	−8.3	−15.5	+61.3	+149.8	−7.0	−5.8	−8.1
"	0.10	−0.0	−6.7	+105.0	+262.2	−6.8	−19.5	+2.6
"	1.0	−6.5	−9.5	+114.5	+285.4	−17.2	−92.0	−0.1
2,2′-B	0.10	−9.7	+1.4	+42.6	+63.3	−6.0	+2.2	+2.4
"	0.30	−5.8	−2.6	+107.2	+140.0	−3.6	−0.8	+5.1
"	1.0	+1.8	+3.2	+109.0	+115.7	−4.4	−4.8	+12.2
"	2.0	−1.4	+2.1	+115.3	+136.2	−2.9	−14.3	

* A = without preincubation.

† B = enzyme preincubated with chelating agent for 10 minutes.

TABLE III

Effects of Chelating Agents on TTC Reduction by Heart Mitochondria

Substrate	Per cent change	
	1,10-P (0.03 mM)	2,2′-B (0.3 mM)
α-Ketoglutarate	−93.9	−87.4
Malate	−59.1	−61.0
Citrate	−37.5	−40.0
Isocitrate	−13.7	−18.6
Succinate	−26.0	−27.8

reported by Mahler and Elowe (5). It is likely that none of these individual enzymes represents the site of the potent mitochondrial inhibition.

Inhibition of TTC Reduction—Reduction of TTC in mitochondrial suspensions occurred with all the substrates employed. Marked inhibition by 0.03 mM 1,10-P and 0.3 mM 2,2′-B was observed with α-ketoglutarate and definite inhibitions, comparable to those observed manometrically, with the other substrates (Table III). A diphasic inhibition, as described in the oxygen uptake, was also found for TTC reduction (Fig. 1, Curve C).

These results indicated the site of inhibition to be between substrate and flavoprotein in the electron transport sequence.

Mitochondrial Oxidation of Added DPNH—The oxygen uptake of mitochondria with DPNH as substrate was only slightly depressed (4 per cent) by 0.03 mM 1,10-P and slightly stimulated (5 per cent) by 0.3 mM 2,2′-B. This was substantiated by the spectrophotometric determination of DPNH oxidation in mitochondria in which only a small depression by 0.03 mM 1,10-P was seen.

TABLE IV

Effect of Fe^{++} and Mn^{++} on Inhibition of Mitochondrial Oxidation of α-Ketoglutarate by 1,10-P and 2,2′-B

Metal ion	Concentration	Per cent change		
		1,10-P (0.03 mM)	Metal ion only	1,10-P + metal ion
	mM			
Fe^{++}	0.01	−100.0		−96.3
"	0.03	−100.0	+23.1	−100.0
"	0.10	−100.0	+37.9	−92.5
"	0.30	−100.0	−12.5	−75.8
"	1.0	−100.0	−28.1	−72.9
Mn^{++}	0.01	−97.2	−51.1	−90.3
"	0.03	−97.2	−64.5	−81.9
"	0.10	−97.2	−66.9	−77.9
"	0.30	−97.2	−65.9	−82.2
		2,2′-B (0.3 mM)		2,2′-B + metal ion
Fe^{++}	0.10	−92.0	+37.9	−98.1
"	0.30	−92.0	−12.5	−73.1
"	1.0	−92.0	−28.1	−66.8
Mn^{++}	0.01	−78.7	−51.1	−87.2
"	0.03	−78.7	−64.5	−82.2
"	0.10	−78.7	−66.9	−77.7
"	0.30	−78.7	−65.9	−69.1
"	1.0	−78.7	−66.7	−65.3

Effects of Metal Ions on Inhibition—The possibility that the chelating agents were combining with a dissociable metal involved in electron transport was tested by addition of several metal ions to determine whether the inhibition might be reduced. A wide range of concentrations (0.0003 to 1.0 mM) of Cu^{++} and Zn^{++} did not alter the inhibitions produced by the chelating agents on the oxidation of α-ketoglutarate. Concentrations of Fe^{++} from one-third to 3 times the concentration of 1,10-P did not alter the inhibition appreciably, but the higher concentrations of Fe^{++} did reduce

the inhibition by 1,10-P and 2,2′-B somewhat (Table IV). Mn^{++} at concentrations equimolar with the chelating agents, or higher, reduced the inhibitions of α-ketoglutarate oxidation despite a rather potent inhibitory action of Mn^{++} alone (Table IV). In fact, when Mn^{++} was present at 0.3 to 1 mM, addition of 2,2′-B produced no further inhibition.

Inhibition by 1,10-P and 2,2′-B in Combination—If 1,10-P and 2,2′-B act on the same metal component, suitable combinations should produce the same reduction in inhibition that was observed when the concentration of each inhibitor alone was increased. This was shown to occur, although with the limited number of combinations tested complete disappearance of the inhibition was not seen. Thus, 0.03 mM 1,10-P alone inhibited α-ketoglutarate oxidation 97.2 per cent, and 0.3 mM 2,2′-B alone inhibited 83.0 per cent; when present together, the inhibition was reduced to 60.9 per cent. When the two chelating agents were present simultaneously, their action was similar to the action of one chelating agent at a higher concentration. Thus it is likely that both these chelating agents are acting at the same site.

TABLE V

Effects of DNP on Inhibition of Mitochondrial Oxidation of α-Ketoglutarate by Chelating Agents

DNP concentration	Per cent change			DNP concentration	Per cent change		
	DNP	DNP + 1,10-P (0.03 mM)	1,10-P*		DNP	DNP + 2,2′-B (0.3 mM)	2,2′-B*
mM				*mM*			
0		−88.6	−88.6	0		−73.1	−73.1
0.01	−18.2	−81.0	−73.7	0.10	−55.3	−41.9	+30.0
0.03	−63.5	−79.1	−40.1	0.30	−54.3	−51.1	+7.0
0.10	−58.3	−61.5	−7.7	1.0	−82.7	−86.9	−13.1

* Effects of chelating agents calculated on the basis of controls containing DNP.

Effects of DNP on Inhibition—Among the various chemical agents tried, DNP alone was found to be effective in reducing the inhibitory action of 1,10-P and 2,2′-B on α-ketoglutarate oxidation as shown in Table V. DNP at the most effective concentration of 0.1 mM reduced the level of inhibition to that of DNP alone or even below; *i.e.*, in the presence of DNP the chelating agents were less inhibitory. Similar reductions by 0.1 mM DNP of the inhibitions of malate (from 89.5 to 45.5 per cent) and pyruvate + malate (from 96.0 to 83.0 per cent) oxidations exerted by the chelating agents were observed. Inhibition of succinate oxidation by 0.1 mM DNP was always greater than the inhibitions by 1,10-P or 2,2′-B; thus no reduction in inhibition could be observed.

P:O Ratio in Presence of 1,10-P—Since DNP, a phosphorylation-uncoupling agent, is effective in reducing the inhibition produced by the chelating agents, the direct effect of 1,10-P on mitochondrial phosphorylative activity was studied. A concentration of 0.3 mM 1,10-P was selected for these studies, since lower concentrations of 1,10-P inhibited the oxygen uptake potently. A definite reduction of the P:O ratio of rat heart mitochondria with α-ketoglutarate as substrate is shown in Table VI. DNP, by comparison, reduced the P:O ratio to 1 approximately.

TABLE VI

Phosphorylation Associated with Oxidation of α-Ketoglutarate

	No. of experiments	1,10-P	P uptake	O_2 uptake	$\frac{P}{O}$
		mM	*μatoms*	*μatoms*	
Rat heart mitochondria	4		12.26	4.55	2.72
" " "	4	0.30	9.22	5.30	1.62
" liver "	1		20.00	5.25	3.81
" " "	1	0.30	20.85	5.77	3.50

TABLE VII

Effects of Chelating Agents on Oxidation of Various Substrates by Liver Mitochondria

Substrate	Per cent change	
	1,10-P (0.03 mM)	2,2′-B (0.3 mM)
α-Ketoglutarate	−3.3	−7.7
Malate	+0.5	+3.0
Succinate	−8.6	−9.6
Pyruvate	−32.4	+40.2
" + malate	−4.1	−5.8

Action of Chelating Agents on Rat Liver Mitochondria—In contrast to the potent inhibitory action of 1,10-P and 2,2′-B on heart mitochondrial oxidations, these agents did not exert appreciable inhibition of the oxidation of various cycle substrates by liver mitochondria (Table VII). That this represents a basic difference between heart and liver mitochondria, not attributable to a difference in the preparation of the mitochondria, was shown by demonstrating the characteristic inhibitions by 1,10-P and 2,2′-B on heart mitochondria prepared by the isotonic sucrose technique. As shown in Table VI, the P:O ratio of rat liver mitochondria was not significantly altered in the presence of 1,10-P. This further illustrates the difference between liver and heart mitochondria.

DISCUSSION

The significant results that have emerged from the present studies are as follows: (*a*) the most sensitive reactions in the cycle to 1,10-P and 2,2′-B are oxidations involving DPN, (*b*) the primary site of inhibition is not on any readily identified component of the electron transport sequence, (*c*) the inhibition is reduced or abolished by increasing concentrations of the chelating agents, (*d*) the inhibition is reduced substantially by DNP, Mn^{++}, and higher concentrations of Fe^{++}, and (*e*) the P:O ratio is reduced from 2.72 to 1.62 in the presence of 0.3 mM 1,10-P.

The inhibitions by 1,10-P and 2,2′-B are similar, as evidenced by the relative potency in inhibiting the oxidation of various cycle substrates, the similar reversal effect at higher concentrations of the chelating agents, and the reduction of inhibition when the most effective inhibitory concentrations of the two chelating agents were used together.

The parallelism between the inhibition of TTC reduction and oxygen uptake would imply that the inhibition is somewhere between the substrate and the flavoprotein as Kuhn (15) and Brodie and Gots (16) have given evidence that a flavoprotein is involved in the reduction of TTC. Steps which are known to be in this segment of the electron transport sequence, such as the α-ketoglutaric or malic dehydrogenase and DPNH cytochrome *c* reductase, and the mitochondrial oxidation of DPNH are not inhibited significantly by the chelating agents. Thus an unknown component of the electron transport chain might be the site of inhibitory action of 1,10-P and 2,2′-B. Non-heme iron has been found in relative abundance in the electron transport system (17). The reduction of 1,10-P and 2,2′-B inhibition by higher concentrations of Fe^{++} is significant inasmuch as $(1,10\text{-P})_3 \cdot FeSO_4$ and $(2,2'\text{-B})_3 \cdot FeSO_4$ exert similar inhibitory actions to those of 1,10-P and 2,2′-B, respectively. The reduction of inhibition by low concentrations of Mn^{++} is very distinct, and Mn^{++} has been reported previously to promote phosphorylation (18). The involvement of a metal component in the inhibition is thus a possibility. The effective reduction of 1,10-P or 2,2′-B inhibition by DNP and the reduction of P:O ratio in the presence of 0.3 mM 1,10-P suggest a connection between a phosphorylative step in the electron transport chain and the inhibition. Since metal has been suggested as a connecting link between phosphorylation and electron transport (19, 20), a common focus of the inhibitory action of the chelating agents may be postulated. One possible site of action could be the oxidation of intramitochondrial DPNH. According to Chance and Williams (21), the oxidation of intramitochondrial DPNH is one of the sites for coupled phosphorylation. The lack of reactants, such as adenosine diphosphate and inorganic phosphate, in the coupled phosphorylation will inhibit the oxidation of intramitochondrial DPNH. Similarly, the 1,10-P

and 2,2′-B inhibition of mitochondrial oxidation may be due to the inhibition of coupled phosphorylation which can be partly released by the addition of DNP.

The reduction in inhibitory potency by increase in concentration of a chelating agent has been observed in studies on the growth of microorganisms. Such an inversion effect has been reported for 8-hydroxyquinoline on micrococci and streptococci (22), diethyldithiocarbamate on *Penicillium* and *Aspergillus* (23), and diethyldithiocarbamate on spore formation of the fungus, *Venturia inaequalis* (24). No definite explanation of such inversion has been made.

SUMMARY

Electron transport in diphosphopyridine nucleotide-linked oxidation systems in rat heart mitochondria was found to be inhibited potently by the chelating agents 1,10-phenanthroline and 2,2′-bipyridine. The major interference of these agents with the operation of the tricarboxylic acid cycle is mediated through this inhibition. Increasing concentrations of the chelating agents reverse this inhibition which may be correlated with previously reported reversals in the depression of growth of microorganisms by chelating agents. The inhibition is reduced by 2,4-dinitrophenol, Mn^{++}, and higher concentrations of Fe^{++}. 1,10-Phenanthroline at a higher concentration did not reduce the oxygen uptake but did reduce the P:O ratio by 1 unit. It is postulated that the chelating agents form a complex with a metal ion which may be involved in phosphorylation coupled to the oxidation of intramitochondrial reduced diphosphopyridine nucleotide.

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ON THE RESTORATION OF FATTY ACID BIOSYNTHESIS AFTER FASTING*

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(Received for publication, October 11, 1957)

The synthesis of fatty acids (1–3) and the individual enzymes concerned with fatty acid metabolism (4) have been studied in detail. It has been observed that the rate of fatty acid synthesis decreases after fasting (5–9), the effects of which on lipogenesis were observed in many tissues such as gut, skin, etc.; the liver, however, appeared to be the most susceptible organ (10, 11).

In the work reported here a substance is described which increases the rate of fatty acid synthesis in cell-free liver preparations obtained from the livers of fasted rats. This material can be isolated from liver and other sources; it has been partially purified and its effect on fatty acid synthesis investigated. It appears that the rate of fat synthesis in liver preparations depends upon the concentration of this material and it is, therefore, suggested that its synthesis by the liver serves the function of a biochemical control mechanism. A preliminary report of these findings has been made (12).

EXPERIMENTAL

Female Sprague-Dawley rats weighing 90 to 100 gm. were used in all experiments. They were kept at 20° and fed a high carbohydrate, fat-free diet *ad libitum* (fed rats). After 4 days on this diet, rats were fasted for 30 hours with access to water (fasted rats). The change of liver weight[1] with the duration of fasting is given in Fig. 1. Both fed and fasted animals were killed by decapitation and the livers immediately excised and chilled in isotonic saline. After blotting, they were transferred onto a stainless steel screen (40 mesh) on the bottom of a flattened tube, about 22 mm. in diameter, and homogenized by a rotating and loosely fitting

* This work was supported by grants from the National Heart Institute, National Institutes of Health, United States Public Health Service.

† Part of this work was carried out while G. N. C. was the recipient of a stipend from the United States Foreign Operations Administration on recommendation of the United States National Academy of Sciences.

[1] Occasionally rats were observed to suffer from an infection which manifested itself by watering eyes, sneezing, and lethargy. Such animals, even after 2 days of fasting, had livers weighing 5 to 5.5 gm. The homogenates from these livers showed only negligible fat synthesis, which could not be increased by liver or yeast extracts.

stainless steel pestle (about 1 mm. clearance). 3 to 4 gm. of liver could thus be homogenized in about 15 seconds.

Incubation Medium—Preliminary experiments were carried out in order to determine the nature and quantity of the components required for maximal fatty acid synthesis in liver homogenates from fed animals.

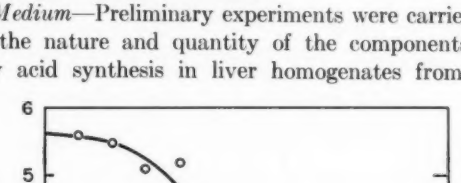

FIG. 1. Weight of liver per 100 gm. of body weight plotted against time of fasting

TABLE I

Incorporation of C^{14} from Acetate and Pyruvate into Fatty Acids by Liver Homogenates from Fed Rats

Additions	Relative isotope concentration*	
	1-C^{14}-Acetate	2-C^{14}-Pyruvate
None	8	15
0.008 M hexose	9	
0.008 " citrate	77	47
0.02 " "	100	100
0.008 " " + 0.00025 M CoA	84	
0.02 " glutamate	71	
0.008 " fumarate	5	
0.008 " fluoroacetate	12	
0.008 " " + 0.008 M fumarate	43	

*Relative isotope concentration of isolated fatty acids. The incubations were carried out as described in Table II, except that the components of the medium were substituted as indicated.

ATP,[2] TPN, CoA, hexose diphosphate, aspartate, ketoglutarate, pyruvate, and lactate were without effect, but succinate, fumarate plus fluoroacetate, glutamate, and particularly citrate increased the rate of fatty acid synthesis in the liver homogenates (Table I). The action of citrate was enhanced

[2] The following abbreviations are used: ATP, adenosine triphosphate; AMP, adenosine monophosphate; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; CoA, coenzyme A; GSH, glutathione.

if the Mg^{++} concentration was also increased, since the effective concentration of this ion is apparently decreased by the complex-forming action of citrate. The optimal citrate and Mg^{++} concentrations are given in the standard medium described below. No consistent increase was observed after the addition of any of the compounds mentioned above if citrate was already present. An optimal concentration of DPN for maximal fatty acid synthesis was found. Incorporation of C^{14}-acetate increased linearly for 3 to 4 hours of incubation and then leveled off. The incorporation of 2-C^{14}-pyruvate was similarly increased by the addition of citrate (Table I). A standardized procedure based on the preliminary results was used in all subsequent experiments (Table II).

Lipide Synthesis in Homogenates from Fed and Fasted Animals—Homogenates of livers from fasted animals incorporated only about one-fourth as much acetate carbon into fatty acids as did those from fed animals, in accordance with previous observations (8, 9). The added acetate nearly saturated the system, so that doubling the acetate concentration increased incorporation only by about 20 per cent. The incorporation of acetate carbon into the unsaponifiable fraction was also reduced by fasting, as has been reported (14). To localize the effect of fasting, liver homogenates from both fed and fasted rats were fractionated by centrifugation at 10,000 $\times$ g for 20 minutes; a particulate fraction sedimented which consisted mainly of mitochondria; the supernatant fluid was decanted. The activities of these fractions alone and after recombination are given in Table II. The supernatant fluids alone from homogenates of fasted and fed animals incorporated nearly identical quantities of acetate carbon. Similarly, the mitochondrial fractions of both kinds of homogenates did not show significant differences. In the recombination experiments only mitochondria from homogenates of fed rats increased the rate of fatty acid synthesis when added to the supernatant fluids from either fasted or fed animals. Microsomal fractions isolated by centrifugation at 100,000 $\times$ g for 30 minutes, if added to the supernatant fluids, were without effect.

To get information on the nature of this effect of mitochondria from fed rats, boiled extracts of liver, liver mitochondria, and yeast were prepared, and their effects on homogenates from fasted and fed rats tested. The boiled extracts seemed to have no effect on the supernatant fluids alone, but increased the rate of fatty acid synthesis in homogenates from fasted animals over 3-fold (Table III). The incorporation of acetate carbon into the unsaponifiable fraction was not affected by the extracts (Table IV). If the boiled extracts were added to homogenates from fed rats, fatty acid synthesis as measured by incorporation of acetate carbon remained either unchanged or was inhibited (Table III). Yeast extract was more inhibitory than liver extract. A similar inhibition in homogenates from fasted animals was observed when the quantity of yeast extract was increased

TABLE II

Incorporation of C^{14}-Acetate into Fatty Acids by Various Liver Homogenate Fractions

Homogenate components from				Relative isotope concentration	
Fed rats		Fasted rats			
Mitochondria	Supernatant fluid	Mitochondria	Supernatant fluid	Average*	Range
−	−	+	−	9	2–14
+	−	−	−	11	4–16
−	−	−	+	26	11–40
−	+	−	−	26	14–39
−	−	+	+	25	9–40
−	+	+	−	24	6–38
+	−	−	+	101	74–116
+	+	−	−	100	

* Five experiments were carried out. 1 gm. of liver was homogenized in 2.5 ml. of a modified Bucher (13) medium containing sucrose, $MgCl_2$, nicotinamide, and phosphate buffer. The homogenate was centrifuged at 700 × *g* for 15 minutes and the inactive pellet consisting of intact cells, debris, and nuclei was discarded. To 2 ml. of the supernatant fluid were added DPN, 1-C^{14}-acetate, citrate, and the desired test material to give a final volume of 2.5 ml. at pH 7.5. The acetate had a specific activity of about 10 μc. of C^{14} per mmole. The final concentrations of the components were as follows: sucrose, 0.096 M; $MgCl_2$, 0.012 M; nicotinamide, 0.022 M; potassium phosphate, 0.035 M; DPN, 0.0008 M; potassium citrate, 0.020 M; potassium 1-C^{14}-acetate, 0.004 M. The mixture was incubated in air for 3 hours at 37° in 25 ml. Erlenmeyer flasks with swirling. After incubation, the contents were added to 2 ml. of diethyleneglycol and 2 ml. of 4 M aqueous KOH and heated at 120° for 1 hour. After extraction of the unsaponifiable material with petroleum ether, the mixtures were acidified to pH 2 with diluted H_2SO_4 and extracted with petroleum ether. The extracts were washed with a 5 per cent solution of acetic acid to dilute the labeled acetate and with water until the washings remained neutral. Control experiments showed the absence of labeled acetate in such extracts. The residues from the petroleum ether extracts were dried on aluminum planchets and weighed, and their radioactivity was determined in a gas flow Geiger counter. The radioactivity was calculated as counts per minute per mg. of fatty acid. The probable counting error was in no case greater than ±5 per cent. In general, the C^{14} concentrations are given relative to the sample containing 0.02 M citrate, which was taken as unity. The actual counts obtained for these reference samples varied between 50 and 150 c.p.m. per mg. in individual experiments with homogenates from fasted rats and represent an incorporation of from 0.02 to 0.06 μmole of acetate carbon. The amounts of fatty acids which were isolated from both fed and fasted rats varied from 1.5 to 2.5 mg. per gm. of liver.

above an optimal value (Table III). A number of compounds were tested for their ability to replace the liver or yeast extracts in increasing the synthesis of fatty acids by homogenates obtained from fasted rats, but no

other compound tested could replace the activity of the extracts (Table V). In the presence of yeast extract the acetate carbon was mainly in the

TABLE III

Effect of Boiled Extracts and Mitochondrial Lysates on Fatty Acid Synthesis in Liver Homogenates

Additions	Relative isotope concentration	
	Fasted animals	Fed animals
None	100	100
0.1 ml. liver extract (fed rat)	140*	115*
0.2 " " " " "	180*	125*
0.4 " " " " "	250*	95*
0.4 " pig liver extract	185	
0.4 " liver extract (fasted rat)	110	
0.4 " mitochondrial extract	150	
0.2–0.8 ml. mitochondrial lysate	130–80	
0.1–1.0 " acid-hydrolyzed liver extract (fed rat)	65–85	
0.1–1.0 ml. ash solution from liver extract (fed rat)	80–95	
0.005 ml. yeast extract	178	
0.1 ml. yeast extract	320	37*
0.2 " " "	160	
0.4 " " "	72	

* Average of several experiments.

Incubation procedure as in Table II. Liver extracts: 10 gm. of fresh liver were dropped into 25 ml. of boiling water and boiled for 2 minutes. The mixture was cooled, and the solid material ground with mortar and pestle. It was made to the original volume with water, boiled again for 3 minutes, cooled, and centrifuged at 700 × *g* for 15 minutes. The pellet was inactive. Boiled extracts from pig liver and rat liver mitochondria were prepared in the same manner. Mitochondria lysates: 1.6 gm. of wet mitochondria were dispersed in 4 ml. of water and left at 4° for 90 minutes. The dispersion was centrifuged at 10,000 × *g* for 30 minutes. Acid hydrolysis: 5 ml. of boiled extract and 1 ml. of 1 N HCl were heated in a boiling water bath for 30 minutes. This treatment destroyed the activity of the extract. Ashing: 2 ml. of boiled extract were ashed in a platinum crucible, the residue was treated with 2 ml. of boiling water, and filtered. Yeast extract: a boiled extract from yeast was prepared in the same manner as the liver extract. It was centrifuged at 20,000 × *g* for 1 hour. The precipitate as well as a fatty layer floating on top was inactive and was discarded.

esterified fatty acid fraction, since extraction before hydrolysis yielded only a small amount of isotope in the unesterified fatty acid fraction. Upon decarboxylation of the isolated fatty acids the ratio of the isotope concentration in the carboxyl group to that of the amine was 2.6 in the absence and 2.1 in the presence of the yeast extract (Table VI).

The aqueous yeast extract was an even better source of the active material than was the liver extract (Table III). It was stable if treated with equal volumes of 0.1 N formic acid either for 3 hours at 37° or 48 hours at

TABLE IV

Effect of Boiled Rat Liver Extract on Acetate Incorporation into Unsaponifiable Fraction of Homogenates from Fasted Animals

Additions	Relative sotope concentration*
None	100
0.1 ml. liver extract	120
0.2 " " "	110
0.3 " " "	75
0.5 " " "	105

* Average of two experiments. Incubation procedure as in Table II.

TABLE V

Effect of Additions on Fat Synthesis in Liver Homogenates from Fasted Rats

Additions	Relative isotope concentration
None	100
0.1 ml. yeast extract	800*
0.4–4 mg. per 100 ml. glycogen	70–120†
0.0008–0.04 M hexose diphosphate	195–5†
0.00004–0.0016 M glycerol	130–80†
0.000026–0.0005 M glycolic acid	70–115†
0.0008 M AMP	88
0.001 M ATP	55
0.0016 M DPN	65
0.00008–0.003 M TPN	70–105†
0.008 M GSH	93
0.0008 M coenzyme A	70
0.000004–0.0025 M cocarboxylase	140–75†
0.00000016–0.0025 M lipoic acid	15–1†
0.000008 M lipoic acid + 0.00008 M cocarboxylase	130
0.000008–0.0008 M lecithin	110–90†

* Average of several experiments.

† Range for several concentrations. Incubation procedure as in Table II.

0°, but it lost its activity in the presence of an equal volume of 1 N formic acid. Similar treatment with 0.001 N ammonia resulted in a loss of activity which was more pronounced at 37°. The active ingredients were not precipitated with ammonium sulfate; they remained in the aqueous phase after extraction with ether or butanol and only about one-third dialyzed through cellophane tubing in 40 hours. Incubation with α- or β-amylase

and pepsin for 2 hours at 37° did not seem to inactivate the material markedly. The active ingredient was removed by charcoal from solution but it was not possible to recover any activity from the charcoal by elution with water, pyridine, or phenol. It did not absorb well on cellulose, diatomaceous earth, or a number of ion exchange resins. However, passage through resin columns increased the specific activity of the material considerably, since large amounts of inactive compounds were retained by the columns.

Purification by Ion Exchange—Dowex 1 formate was prepared (17) by suspending the commercial resin (chloride form) in water to eliminate the extremely fine and coarse particles. The resin was treated with a 3 M aqueous solution of sodium formate until no more chloride ion could be

TABLE VI

Distribution of Acetate Carbon in Fatty Acids from Liver Homogenates of Fasted Rats

Fraction	Relative isotope concentration*	
	Without yeast extract	With yeast extract
Isolated fatty acids	100	100
Carboxyl carbon	240	202
Decarboxylation residue	89	98

* Counted as infinitely thick samples of $BaCO_3$. Incubation procedure as in Table II.

The isolated fatty acids were diluted with ordinary palmitic acid and decarboxylated with sodium azide in sulfuric acid (15). The evolved carbon dioxide was precipitated as barium carbonate, as was the carbon dioxide obtained by dry combustion of the remaining amine and of a sample of the fatty acids themselves. All samples were counted as infinitely thick layers in a gas flow counter (16).

removed, and then with water. It was poured as a thin slurry into a column and washed, before use, with 5 to 10 bed volumes of a mixture of 6 N formic acid and 1 M sodium formate (1:1), with several bed volumes of 88 per cent formic acid, and finally with water until the effluent was neutral.

Dowex 50 ammonium resin was prepared by treating the commercial product with 1 N HCl to remove any sodium ions and with water until neutral. This was followed by treatment with 1 N aqueous ammonia until no more ammonium ions could be absorbed by the resin, and by washing with water to neutrality.

10 ml. of centrifuged yeast extract were concentrated 5-fold *in vacuo* and added to a Dowex 1 formate column (0.8 × 22 cm.), and the column was washed with water until the active material was eluted. It emerged in the 50 and 65 ml. fraction. The first 50 ml. of eluate contained a large

amount of inactive material and other inactive material remained on the column. About 2.5 per cent of the solute was recovered in the active fraction. The material was rechromatographed on an identical Dowex 1 formate column with about 14 per cent recovery of solute containing nearly all the activity and then passed through a Dowex 50 ammonium column (0.8 × 10 cm.). The active material emerged in the first eluate and some inactive solute remained adsorbed. The over-all purification achieved by this procedure compared to the original yeast extract was about 400-fold. The total activity recovered in the partially purified extract was the same as or somewhat larger than the activity of the crude extract in spite of the losses inherent in any purification procedure (Table VII).

TABLE VII

Effect of Fractions Eluted from Dowex 1 Formate and Dowex 50 Ammonium Columns on Incorporation of Acetate Carbon in Homogenates from Fasted Rats

Additions	Relative isotope concentration	
	Dowex 1	Dowex 50
None	100	100
0.01 ml. yeast extract	915	80
0.01 " Fraction 1	390	2050
0.01 " " 2	380	220
0.01 " " 3	230	180
0.01 " " 4	185	95
0.01 " " 5	3100	115
0.01 " " 6	940	185
0.01 " " 7	320	125
0.01 " " 8	385	100

Incubation procedure as in Table II.

DISCUSSION

Harrison (18) studied the effect of fasting on the composition of the liver. It was found here that the rate of lipide synthesis decreased approximately with the time of fasting and with the decrease in relative liver weight (Fig. 1). The fatty acid content of liver homogenates was considerably smaller than that of whole liver, but no consistent differences between homogenates from fasted and fed animals were observed.

Fasting decreased the rate of synthesis of fatty acid as well as that of cholesterol (14). This effect of fasting has been reported for rat liver slices *in vitro* (19) and in cell-free rat liver homogenates (12). Similar results were obtained with pigeon liver preparations *in vitro* (20, 21). The duration of fasting determined the rate of acetate carbon incorporation into the fatty acids. The rate remained at a constant level for about

6 hours and then decreased until after 18 to 24 hours it reached a value of one-fifth to one-tenth of the original rate (8). It had been shown that, upon feeding of a single dose of carbohydrate but not of fat or protein, the liver regained the capacity to synthesize fats in about 5 to 6 hours, even after extended periods of fasting (8). After carbohydrate feeding the rate was observed to increase temporarily even above the non-fasting rate. It follows, therefore, that, upon carbohydrate feeding, the liver can synthesize from the materials already present in the animal the missing substance or substances that permit the complex fatty acid synthesizing system to operate at maximal rate.

The presence of intact mitochondria was required for maximal acetate carbon incorporation (Table II), and, except for added DPN and citrate, no other coenzymes or substrates consistently improved the rate of acetate incorporation. These observations are in agreement with the results of Dituri *et al.* (22), who also showed that homogenization of rat liver in the presence of electrolytes rendered soluble the mitochondrial enzymes partaking in fatty acid synthesis. This observation most probably accounts for the results of Porter and Tietz (23) and of Langdon (24), who found that, after homogenization in buffer, fatty acid synthesis occurred only in the supernatant fluid and was not increased by adding mitochondria. Shaw *et al.* (25) reported that for incorporation of pyruvate carbon the addition of butyryl coenzyme A to mitochondria can, at least in part, replace the requirement for the supernatant fluid. It would, therefore, appear very likely that in the intact cell both the fatty acid oxidizing and the synthesizing enzymes were contained in the mitochondria and that these enzymes were, at least in part, extracted during destruction of cell integrity in ionic surroundings.

Further, the difference between homogenates from fed and fasted animals resided exclusively in the mitochondria (Table II). Not only was the rate of acetate incorporation in supernatant fluids from fed and fasted animals identical, but the addition of mitochondria to these supernatant fluids gave the following results: The addition of mitochondria from fasted rats was without effect, whereas the addition of mitochondria from fed animals increased the synthesis about 4-fold. Both kinds of mitochondria were quite inactive by themselves. The incorporation of acetate carbon into fatty acids by homogenates from fasted rats could be increased several-fold if boiled extracts from liver, mitochondria, or yeast were added (Table III). Such extracts had no effect on the supernatant fluid in the absence of mitochondria, and extracts similarly prepared from livers of fasted animals were inactive. In contrast to previous experiments with pigeon liver homogenates (20, 21), it was found here that glycogen, hexose diphosphate, or other glycolytic intermediates and coenzymes could not replace the liver or yeast extracts.

It is at present not clear whether inhibition by high concentrations of yeast extract was an inherent property of the active material in these extracts or whether the extracts contained an antagonist which at higher concentrations interfered with fatty acid synthesis. Since the active ingredient could be obtained from the livers of fed rats, a liver homogenate prepared from fed animals apparently already contained an optimal concentration of this material. It seemed that the purified material was less inhibiting than the crude yeast or liver extracts. A given quantity of purified material, with constant activity calculated relative to the original yeast extract, gave the same increase of acetate incorporation. However, more of the purified substance could be added before synthesis became inhibited, and thus a higher absolute rate of fat synthesis was obtainable.

The active material apparently is devoid of charged groups since it could not be absorbed on either basic or acidic ion exchange resins. It was precipitated by alcohol and was not extractable by organic solvents; these results indicate a large number of hydrophilic groups. Since it dialyzed, albeit slowly, through cellophane membranes, its molecular weight can not be very high. It is possible that the material aggregates on purification since no repulsive charges appear to be present on the surface of the molecule. Its solubility resembled that of glycogen, but glycogen could not substitute for it (Table V). 1 γ of the purest material obtained so far was estimated to permit the incorporation of 1 μmole of acetate carbon in the presence of a homogenate obtained from 1 gm. of rat liver incubated for 3 hours. The determination of the composition of this material is at present being undertaken.

Rittenberg and Bloch (3) found that the carbon atoms of acetic acid were uniformly distributed along the fatty acid chain. Such a uniform distribution was also observed in fatty acids synthesized by pigeon liver preparations *in vitro* (26). However, in experiments of Zabin (19) with fasted rats *in vivo*, excess acetate carbon was found in the carboxyl group, and in slices of rat liver *in vitro* the excess isotope in the carboxyl position varied inversely in proportion to the rate of fat synthesis in the individual experiments. In recent experiments of Porter and Tietz (23) with purified pigeon liver preparations, the major fraction of the labeled carbon was found in the C_{14} and C_{16} acids, and the carboxyl group contained only slightly less than the expected amount of isotope; these results indicate nearly uniform incorporation into the whole chain.

In the experiments reported here, the fatty acids synthesized by homogenates from livers of fasted rats contained more isotopic carbon in the carboxyl group than twice that in the remainder of the carbon chain (Table VI); this result indicates that, in part, preformed fatty acids were elongated by 1 acetate unit. In contrast, in the homogenates supplemented

with yeast extract, the carboxyl group contained exactly twice the average isotope concentration, as would be expected for total synthesis. Since liver homogenates contained much less fat than slices, and thus a lesser amount of fatty acid available as substrate for elongation, it was not surprising that the excess carboxyl isotope observed in these experiments was smaller than that found in the slice experiments (19).

The inhibition of acetate incorporation by lipoic acid at very low concentrations and the reversal of this inhibition by cocarboxylase (Table III) have so far not been further investigated. This observation differed from the results obtained by Porter *et al.* (27) in pigeon liver preparations.

The concentrations of the enzymes concerned with fatty acid synthesis and of their cofactors in the liver were apparently not changed by fasting since addition of liver or yeast extract immediately restored fatty acid synthesis. It seems clear, therefore, that the active ingredient contained in these extracts acted indirectly to control the rate of synthesis. It was observed that acetate oxidation was not increased in liver slices from fasted rats (9), and since acetate was present in non-limiting quantities in the experiments described here, it seems unlikely that the yeast extract blocked acetate oxidation. Whereas the mechanism of action remains at present unknown, it appears that there are the following possibilities: either fatty acid oxidation is inhibited and the concentrations of intermediates from carbohydrate breakdown increase until conditions favorable for fatty acid synthesis are reached, or the interaction of a critical intermediate (a reduced hydrogen carrier might serve as such an example) with the fatty acid synthesis complex is promoted. Experiments carried out to investigate this mechanism have not yet given conclusive results.

SUMMARY

A factor was found to be present in the liver of normal rats and pigs and in yeast which, when added to a liver homogenate from fasted rats, increased the rate of incorporation of acetate carbon into fatty acids. Up to a 30-fold increase was obtained with material from yeast which had been purified approximately 400-fold and appeared to act in catalytic amounts.

The substance lost its activity if treated with 1 N hydrochloric acid, was stable in 0.1 N formic acid, and was partly destroyed above pH 9. Although it did not seem to contain free acidic or basic groups, it was soluble only in water. The concentration of this material in the liver seemed to depend upon the nutritional state of the animal and to control the rate of fatty acid synthesis in this organ.

The implications of this finding relevant to the biosynthesis of fatty acids were discussed.

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A NEW ETHANOLAMINE-CONTAINING LIPIDE FROM EGG YOLK*

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(Received for publication, November 27, 1957)

Recent studies of egg yolk lipides and lipoproteins, and in particular the results of the chromatographic procedures of Lea and coworkers (1–5), have caused renewed interest in egg yolk, which many years ago was considered the most important source of phosphatides for both scientific and commercial purposes. Lea and Rhodes have extensively studied the phospholipide content of egg yolk (1–3), whereas Lea and Hawke (4, 5) have worked mainly on the lipoproteins. Despite the attention given to egg yolk lipides, very few data are available on the sphingolipide constituents. In undertaking a study of this fraction, we prepared a considerable quantity of crude egg yolk phosphatide[1] and subjected this material to the mild alkaline hydrolysis procedure of Schmidt *et al.* (6). The resulting crude sphingolipide fraction contained both cerebroside and sphingomyelin as determined by paper chromatography of the intact lipide mixture and of its hydrolysis products. Glycerol, inositol, and their phosphates were not detected in the hydrolysates but the intact crude sphingolipide mixture gave a positive ninhydrin test and an acid hydrolysate contained ethanolamine and ethanolamine phosphate. Since none of the known phospholipides (except sphingomyelin) is stable to mild alkaline and acid hydrolysis (6), these unexpected observations could reasonably be interpreted as indicating the presence in egg yolk lipides of the ethanolamine analogue of sphingomyelin (I) (tentatively designated as sphingoethanolamine).

This substance has not been isolated from natural sources, although there are numerous indications in the literature of its possible existence.

* This work was supported by a generous grant from the Procter and Gamble Company and in part by a research grant (No. B574-C3) from the National Institutes of Health, United States Public Health Service. A preliminary report of this work was presented (*Federation Proc.*, **16**, 817 (1957)).

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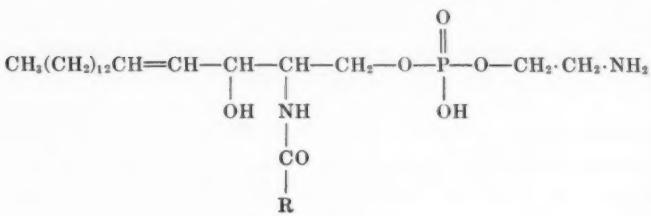
[1] The starting material for these studies was 50 pounds of "egg oil" obtained through the courtesy of the VioBin Corporation, Monticello, Illinois. The authors wish to express their appreciation to Mr. Ezra Levin for providing this material.



(I)

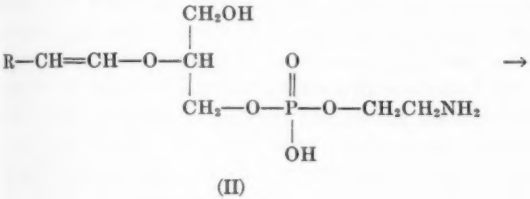
Thus Dawson (7) found that the "alkali-stable" cerebral lipides gave a choline to phosphorus ratio of less than 1, indicating the presence of sphingolipides other than sphingomyelin. Brante (8) and Edgar (9, 10) have reported that certain cephalin fractions isolated from cerebral tissue contained alkali- and acid-stable lipide phosphorus although no choline was present. More recently Weiss (11) has observed a ninhydrin-positive component of the sphingolipide fraction obtained by column chromatography of brain lipides. And finally, a novel, alkali-stable lipide of a different type has been reported by Mallov *et al.* (12), who obtained sphingomyelin phosphorus values in excess of the total sphingosine content on sphingolipide fractions prepared from heart lipides by the Schmidt procedure (6). In view of these indications of the possible existence of a novel "alkali-stable" lipide, it seemed of interest to attempt the isolation of the ninhydrin-positive constituent.

The crude lipide fraction obtained from the mild alkaline hydrolysis of egg phosphatide was fractionated on a silicic acid column by eluting with increasing concentrations of methanol in chloroform. Four main fractions containing ceramide, cerebroside, ethanolamine-lipide plus cerebroside, and sphingomyelin were eluted by 5, 15, 55, and 70 to 90 per cent methanol, respectively, in chloroform.[2] Further fractionation of the mixture of ethanolamine-lipide and cerebroside by column chromatography could not be achieved either on silicic acid or on alumina columns. The two components were separated by chromatography on silicic acid-impregnated paper (13) with chloroform-methanol as solvent, but at this time a more promising method for the purification of the crude ethanolamine-lipide was obtained by a combination of solvent and column procedures. Treatment of the crude sphingolipide mixture with pyridine removed the bulk of the cerebroside as the soluble fraction, which was ninhydrin-negative. Treatment of the pyridine-insoluble residue with cold acetic acid left an insoluble material which contained mainly cerebroside. Addition of acetone to the acetic acid solution precipitated a ninhydrin-positive frac-

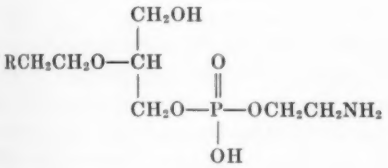
[2] These results closely parallel those reported by Weiss (11) in the fractionation of brain sphingolipides.

tion which still contained a little cerebroside. However, further additions of acetone gave three more fractions, all cerebroside-free, the first two being strongly ninhydrin-positive. Application of the silicic acid column procedure to these fractions gave 235 mg. of the ethanolamine-lipide uncontaminated with cerebroside.

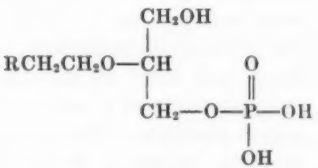
To our surprise, this cerebroside-free material showed no long chain base nitrogen by the McKibbin-Taylor procedure (14); this result ruled out the sphingoethanolamine structure (I). No glycerol or glycerol phosphate was detected by paper chromatography of acid or alkaline hydrolysates, but the chloroform-soluble extract from the McKibbin-Taylor hydrolysis contained a ninhydrin-negative phosphatidic acid-like component. The marked stability of this material excluded the possible presence of an ester, acetal, or vinyl ether group. Several types of structure were considered but the most interesting possibility was that the ethanolamine-lipide contained an ether linkage and was analogous in structure to the saturated ether (III) obtained by Rapport *et al.* (15) by reduction of lysophosphatidal ethanolamine (II). The phosphatidic acid would then have Structure IV.



(II)



(III)



(IV)

This view was supported by the following evidence: (1) Glycerol determinations and elementary analytical data were in satisfactory agreement with the postulated structure. (2) Hydrolysis with acid or alkali gave as the main products ethanolamine and a chloroform-soluble phosphatidic acid. Little or no water-soluble phosphate was produced. (3) The infrared spectrum of the ethanolamine-lipide showed a strong polymethylene peak (1470 cm.$^{-1}$) but was devoid of amide or ester peaks. Furthermore, the spectrum contained a definite peak at 1118 cm.$^{-1}$ which is characteristic of a number of long chain ether derivatives of glycerol

(range 1114 to 1119 cm.$^{-1}$), including batyl and chimyl alcohols and their diacetates.

To substantiate further the postulated structure, advantage was taken of the observation of Malkin and coworkers (16) that phosphate esters are cleaved by a refluxing acetic anhydride-acetic acid mixture. Application of this procedure to the ethanolamine-lipide readily yielded a phosphorus-free material, the solubility and melting point behavior of which were closely similar to those of the diacetates of glycerol α- and β-octadecyl ethers[3] (17, 18). The infrared spectra of the three substances proved to be identical and the presence of a strong ether band at 1118 cm.$^{-1}$ in each affords conclusive proof of the presence of the glycerol ether structure.

These data provided no evidence on the position of attachment of the ether moiety to glycerol, nor does consideration of the possible biosynthetic relationships between the ethanolamine-lipide and the known naturally occurring long chain ethers of glycerol provide any indication of the position of attachment. Naturally occurring long chain ethers of glycerol fall into two categories: the saturated ethers, batyl and chimyl alcohols (octadecyl and hexadecyl glycerol ether, respectively), in which the point of attachment is at the α position, and the vinyl ethers which are present in the plasmalogens, in which the point of attachment is in contention. Rapport and Franzl (19) have provided strong enzymatic evidence for the β attachment of the vinyl ether group, but recently chemical evidence for the α-vinyl ether structure also has been presented (20). Since there was no basis for preference of either structure, it was desirable to investigate this point further. The glycerol α-ether structure was conclusively established by periodate oxidation and by proton magnetic resonance studies. The glycerol ether diacetate, obtained by degradation of the ethanolamine-lipide, and batyl alcohol diacetate upon hydrolysis and treatment with periodate reduced 1 mole of reagent, whereas a synthetic β-ether did not reduce periodate. This result was confirmed by comparison of the proton magnetic resonance spectra of the degradation product (diacetate) with those of the following compounds: the diacetates of α- and β-octadecyl glycerol ethers, α,β-diacetyl-α'-stearin,[3] α,α'-diacetyl-β-stearin,[3] and n-propyl hexadecyl ether. The chemical shifts obtained, expressed in cycles per second relative to water, are shown in Table I and Fig. 1. The assignments made for the characteristic shifts are given in Fig. 1. The validity of these assignments is substantiated by the correspondence of the type (singlet, doublet, triplet, etc.) and relative intensities of the bands with those predicted (Table II) from the number and location of like hydrogen atoms.

[3] The authors gratefully acknowledge samples of various α- and β-glycerol ethers from Dr. Erich Baer, Dr. T. Malkin, and Dr. P. E. Verkade, and of the stearyl diacetins from Dr. William Griebstein of the Procter and Gamble Company.

The identity of the spectra of synthetic α-octadecyl glycerol ether diacetate and of the degradation product, together with the assignment of the chemical shifts (Fig. 1), showed beyond all doubt that the two compounds have identical structures.

In view of these results, the ethanolamine-lipide and the derived phosphatidic acid are assigned Structures V and VI, respectively. (It is assumed that the phosphorylethanolamine group is attached in the α position by analogy with the known phosphatides of similar composition. However, this point will be confirmed when larger amounts of material become available.)

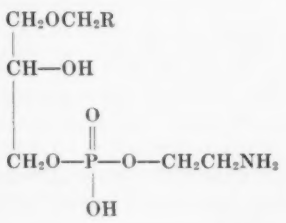
TABLE I

Proton Magnetic Resonance Spectra of Glycerol Derivatives

Resonance bands in cycles per second relative to water.

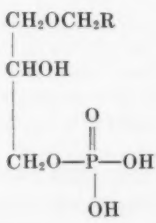
Compound	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6
n-Propyl hexadecyl ether			+56 (t)		+140	+155
β-Stearyl α,α′-diacetate	−20 (qt)	+16 (d)		+104 (s)	+137	+151
α′-Stearyl α,β-diacetate	−23 "	+16 "		+105 "	+138	+154
α-Octadecyl glycerol ether diacetate	−19 "	+20 (d*)	+51 (d)	+108 "	+141	+156
β-Octadecyl glycerol ether diacetate		+24 (d)	+46 (t + qt)	+107 "	+138	+153
Degradation product (diacetate)	−21 (qt)	+16 (d*)	+50 (t)	+106 "	+140	+155

Resonance bands marked s, d, t, qt appeared as singlets, doublets, triplets, and quintuples, respectively. d* signified two superimposed doublets. Bands not marked appeared as single unresolved bands, or multiplets.



R = $C_{17}H_{35}$

(V)



(VI)

One property of the ethanolamine-lipide, namely its stability to hydrolysis in the Schmidt procedure (6), deserves further comment. A

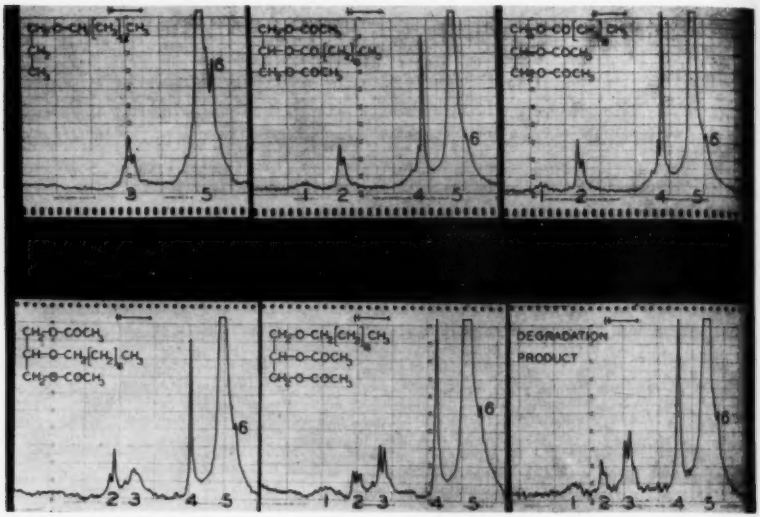


FIG. 1. Proton magnetic resonance spectra of glycerol derivatives. The cycles per second shifts, calculated against water as the standard, are given in Table I and the assignment of the numbered peaks is given below. Band 1, a quintuplet arising from the lone β-hydrogen atom. Of the compounds studied, only those with a β-ester group showed this proton resonance. This result is anticipated by the greater inductive unshielding effect of the ester group than of that of the ether oxygen; the proton resonance of the β-hydrogen thus occurs at a lower field in the compounds with a β-ester than with a β-ether group; Band 2, a doublet in α,α'-diacetyl-β-stearin, α,β-diacetyl-α'-stearin, and β-octadecyl glycerol ether diacetate arising from the four equivalent α-CH_2 hydrogens. In α-octadecyl glycerol ether diacetate, it is composed of two doublets arising from the two non-equivalent hydrogens on the α'-carbon atom; Band 3, this arises from the hydrogens adjacent to the ether oxygen. It occurs as a triplet in *n*-propyl octadecyl ether and β-octadecyl glycerol ether diacetate, and a superimposed doublet and triplet in α-octadecyl ether diacetate; Band 4, the shift (singlet) due to the hydrogens of the ester methyl groups. The shoulders on the left side of this band in the second and third spectra are due to the methylene group α to the ester carbonyl in the long chain; Band 5, proton resonance of the hydrogen atoms in the polymethylene chain; Band 6, the shift due to the hydrogens of the methyl group in the polymethylene chain.

number of compounds containing the phosphate diester group with a free hydroxyl group on the adjacent carbon are readily cleaved by mild alkaline reagents and this is true in the case of glycerylphosphorylcholine. However, Schmidt *et al.* (21) have shown that glycerylphosphorylethanolamine

is moderately stable toward alkali (treatment with 1 N sodium hydroxide for 30 minutes at 100° gave incomplete hydrolysis), so that the stability of the ethanolamine-lipide in 1 N alkali at 37° may not be surprising. Fortunately, in Malkin's laboratory an excellent model compound (VII) had been prepared synthetically and we are grateful to Dr. Malkin for making a sample available to us. This substance under the Schmidt (6) conditions

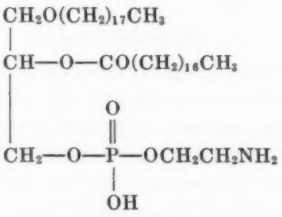
TABLE II

Predicted Relative Intensities of Proton Magnetic Resonance Bands of Glycerol Derivatives

	β-Octadecyl glycerol ether diacetate					α-Octadecyl glycerol ether diacetate				
Resonance band No.	5	4	3	2	1	5	4	3	2	1
Predicted relative intensity	30	6	3	4		30	6	4	2	1
Predicted band type		s	t + qt	d			s	d	d*	qt

The symbols s, t, d, qt, and d* are explained in Table I.

lost the ester group but only a small fraction of the ethanolamine was cleaved off.



(VII)

Thus the behavior of the ether phosphatide is similar to that of glycerylphosphorylethanolamine.

It seems entirely possible that the anomalous behavior of lipides reported by others may result from the general occurrence of similar ether phosphatides. Such compounds would behave in the same way as "cephalin B" of Brante (8), and would account for the high phosphorus values of Mallov *et al.* (12) and the low choline to phosphorus ratios of Dawson (7). In our laboratories, brain sphingolipides (purified by the Schmidt procedure) have given ninhydrin and glycerol determinations, indicating the presence of 1 to 2 per cent of a glycerophosphatide capable of withstanding the alkaline and acidic hydrolysis procedure. The sphingolipides (sphingomyelin, cere-

broside, ceramide, etc.) are stable toward mild alkaline treatment and the material obtained by the alkaline saponification procedure of Schmidt *et al.* (6) has been designated, usually, as the sphingolipide fraction. The discovery of a glycerol ether phosphatide in this fraction makes it undesirable to continue this practice. The term "alkali-stable" lipides might be a more appropriate designation for this fraction.

The discovery of an ether glycerophosphatide in egg yolk raises several interesting questions. What is the substance originally present in egg yolk lipide? In view of the hydrolysis procedure employed, the ethanolamine-lipide actually isolated might well have arisen by degradation of an esterified derivative (VII). We are now attempting to isolate the original lipide by non-hydrolytic procedures in order to answer this question. The distribution of ether phosphatides in animal and plant tissues and their metabolic origin and possible physiological effects (including thromboplastic activity) pose interesting problems. Karnovsky *et al.* (22, 23) have investigated the distribution and metabolism of glycerol ethers in the starfish and Karnovsky (24) has reported evidence for the possible existence of glycerol ether phospholipides in this marine animal. Bergström and Blomstrand (25) have reported studies on the absorption, distribution, and metabolism of chimyl alcohol in the rat. Extension of such experiments should clarify possible metabolic interrelationships of the glycerol ethers, the corresponding phospholipides, and the acetal phospholipides.

EXPERIMENTAL

Preparation of Crude Sphingolipide from Egg Yolk—Egg yolk phosphatide was obtained by exhaustive extraction of egg oil (10 kilos) with acetone. The dried insoluble phosphatide (about 1947 gm.) gave the following analytical figures: total N 2.17, total P 3.13, LCB N[4] 0.078.

This phosphatide was hydrolyzed by the mild alkaline procedure of Schmidt *et al.* (6). 100 gm. of phosphatide were suspended in 2500 ml. of 1 N KOH and the mixture was shaken for 24 hours at 37°. The emulsion was neutralized with 500 ml. of 5 N HCl and brought to pH 1 with hydrochloric and formic acids. After standing for 1 hour in order to destroy acetal lipides, 5 liters of acetone were added and the solution was centrifuged after the fatty acids had dissolved. The residue was washed with 2 per cent aqueous hydrochloric acid-acetone (2:4), then with acetone, and was filtered and dried over P_2O_5 in a vacuum desiccator. From a total of 1215 gm. of phosphatide, 20.9 gm. (1.7 per cent) of crude sphingolipide fraction were obtained. This material, upon analysis, was as follows: total N 2.73, total P 1.8, LCB N[4] 1.06. It is obvious from these data that a considerable proportion of sphingolipide was lost in the supernatant solutions.

[4] Long chain base nitrogen as determined by the McKibbin-Taylor procedure.

Paper Chromatography—Lipide samples (10 to 50 mg.) were hydrolyzed in 1 to 5 ml. of acid or base. The insoluble residue was extracted into chloroform and the water-soluble phase was neutralized and lyophilized. The water-soluble hydrolysis products were chromatographed on Whatman No. 540 acid-washed paper in isopropanol-acetic acid-water (3:1:1). Intact lipides and some chloroform-soluble materials were chromatographed on Whatman No. 3 paper impregnated with silicic acid according to the method of Lea, Rhodes, and Stoll (13).

Reagents—Papers, after development and removal of solvent, were sprayed with ninhydrin (0.5 per cent w/v) in pyridine and heated for 5 minutes at 95° to detect substances containing free amino groups. Choline was detected by spraying with 2 per cent aqueous phosphomolybdic acid, followed by washing and reduction to molybdenum blue with acid stannous chloride (13). This spray can be used after the ninhydrin reagent with good results. Phosphorus was detected by spraying with the Hanes and Isherwood reagent (26), followed by exposure to bright sunlight or ultraviolet light. Bright blue spots were obtained, quite different from the greenish blue inorganic phosphate spots.

Column Chromatography—Silicic acid columns were prepared essentially as described by Lea, Rhodes, and Stoll (13) by mixing a slurry of silicic acid (Mallinckrodt's silicic acid, reagent 100 mesh suitable for chromatographic analyses) in chloroform-methanol (4:1), pouring it into a column of 2.2 cm. diameter, and washing the column, when settled, with the chloroform-methanol mixture. Finally the column was washed with chloroform until transparent. The crude sphingolipide was added in chloroform as a thin band and eluted first with chloroform and then with mixtures of 5, 15, 35, 55, 70, and 90 per cent methanol-chloroform, at least two "hold-up" volumes of each solvent mixture being used. Generally about 350 mg. of sample were used per 40 gm. of silicic acid. However, much more material can be applied, depending on the content of the various sphingolipides present and the degree of separation required. Fractions of about 5 ml. were collected with an automatic fraction collector and appropriate fractions were combined and evaporated. 3 to 5 mg. portions of each solid fraction were dissolved in 10 ml. of chloroform or chloroform-methanol and 1 and 3 ml. aliquots were evaporated for the ninhydrin (1, 2) and anthrone (galactose) (27) determinations, respectively. The infrared spectra of the various fractions proved helpful in determining the type of lipide structure. Typical column results are given later in the description of the isolation of the ethanolamine-lipide.

Preparation of Ethanolamine-Lipide from Crude Sphingolipide—Extraction of the crude sphingolipide (20 gm.) with pyridine at room temperature removed most of the cerebroside as a soluble fraction (3.65 gm.), which gave an anthrone value of 15 per cent (cerebroside = galactose per cent ×

4.55) but was ninhydrin-negative. The pyridine-insoluble fraction was suspended in acetic acid at room temperature to remove further cerebroside as the insoluble residue (2.32 gm.). The acetic acid solution was evaporated to 50 ml. and acetone was added to precipitate Fraction A_1 (3.45 gm.). This material still contained some cerebroside (anthrone 7 per cent), but was ninhydrin-positive. Further additions of acetone yielded three more fractions, A_2, A_3, and A_4, all cerebroside-free. Fraction A_2, 2.05 gm., strongly ninhydrin-positive (glycerol 2.86 per cent); Fraction A_3, 1.41 gm.,

TABLE III

Fractionation of Crude Ethanolamine-Lipide on Silicic Acid

395 mg. of Fraction A_2 were chromatographed over a 35 gm. silicic acid column. The eluate was collected in 5 to 6 ml. fractions.

Tube No.	Solvent*	Fraction No.	Weight	Amino N†	Anthrone‡
			mg.	*per cent*	*per cent*
6–14	0				
15–17	8	1	2.0		3.5
18–26		2	10.7	0.27	0.0
27–28	20				
29–47		3	7.2	0.52	1.0
48–56	35	4	2.4	0.97	3.7
59–77	55	5	41.3	3.00	0.0
79–96	70	6	113.7	0.10	0.9
97–101		7	101.7	0.10	0.0
104–110	90	8	42.4	0.10	0.4
111–135		9	55.2		
Total.....			376.6		

* The numbers give the percentage of methanol in chloroform.
† Calculated against a phosphatidylethanolamine standard.
‡ Calculated as galactose.

strongly ninhydrin-positive; Fraction A_4, 4.32 gm., ninhydrin-negative. Passage of Fractions A_2 and A_3 over silicic acid columns yielded the cerebroside-free ethanolamine-lipide. Typical column results are given in Table III.

From Fractions A_2 (1.02 gm.) and A_3 (1.4 gm.), 235 mg. (about 10 per cent) of the ethanolamine-lipide were obtained by column chromatography. The analyses of the combined fractions were in fair agreement with a batyl, or chimyl, alcohol phosphorylethanolamine.

	$C_{23}H_{50}O_6NP$.	
Calculated.	(batyl).	C 59.10, H 10.71, N 3.00, P 6.64, glycerol 19.7
"	(chimyl).	" 57.4, " 10.48, " 3.19, " 6.06, " 20.9

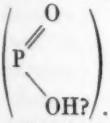
$C_{21}H_{46}O_6NP$.

Found. C 57.37, H 10.08, N 3.24, P 6.24, glycerol 18.2
LCB N^4 0.11; N:P = 1.15; anthrone (as galactose) 0.0

The melting behavior of this material was unusual. Slight sintering began at 140–150° and a mass of tiny liquid crystals appeared at 175°. These melted between 180–190° but almost immediately new larger crystals appeared which did not melt until 206–210°.

Hydrolysis of Ethanolamine-Lipide with Aqueous Sulfuric Acid—50 mg. of the ethanolamine-lipide were refluxed in 5 ml. of 1 N H_2SO_4 for 40 minutes. The aqueous phase was decanted, and the residue extracted into chloroform. The aqueous solution contained mainly ethanolamine plus a slight amount of ethanolamine phosphate. The chloroform-soluble material was chromatographed as a line on silicic acid-impregnated paper in chloroform-methanol (4:1) and small strips were sprayed with the ninhydrin and phosphate reagents. The main spot (R_F 0.91) was strongly phosphate-positive and ninhydrin-negative. Unfortunately, unchanged starting material (R_F 0.80, ninhydrin-positive) was also present and contaminated the phosphatidic acid eluted from the paper. However, the solubility properties and general behavior are consistent with those described for synthetic batyl alcohol phosphates (28).

Hydrolysis of Ethanolamine-Lipides with Barium Hydroxide—The ethanolamine-lipide (15 mg.) was treated with 10 ml. of saturated $Ba(OH)_2$ solution under reflux. After 6 hours the cooled reaction mixture was acidified and treated with chloroform. The chloroform extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated. The residue (10 mg.) of glycerol ether phosphate was characterized by paper chromatography and by an infrared spectrum. In chloroform solution, absorption maxima appeared at 2930 and 2860 $cm.^{-1}$ (CH_3, CH_2, and CH stretch), 1730 $cm.^{-1}$ (broad and weak, acid phosphorus compound?), 1470 $cm.^{-1}$ ($-CH_2-$ deformation), 1384 $cm.^{-1}$ ($C-CH_3$ deformation), 1265 $cm.^{-1}$ (very strong and sharp, P=O (free) stretch). There were also very broad, strong bands at 1090 $cm.^{-1}$, 1015 $cm.^{-1}$, 865 $cm.^{-1}$, and 810 $cm.^{-1}$, and very broad but shallow absorption in the region 2600 $cm.^{-1}$ to 3400 $cm.^{-1}$



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Acetolysis of Ethanolamine-Lipide—The procedure of Malkin and coworkers (16) was slightly modified. The ethanolamine-lipide (20 mg.) was refluxed with a mixture of acetic anhydride and acetic acid (5 ml. 2:3) for 8.5 hours. The cooled reaction mixture was lyophilized and the residue shaken with a mixture of water and ether (1:4). The ether layer was

washed twice with small quantites of water, dried over anhydrous sodium sulfate, and evaporated to a waxy solid. The infrared and *n-m-r* spectra of this crude diacetate were identical with those of α-octadecyl glycerol ether diacetate (see below).

The aqueous layer and washings were combined and lyophilized. Paper chromatography of the residue showed the presence of ethanolamine, a little ethanolamine phosphate, and much inorganic phosphate.

Periodate Oxidations—The acetolysis product (15 mg.) was dissolved in 0.5 ml. of 1 N alcoholic KOH and the solution was allowed to stand at room temperature overnight. Water was added and the emulsion was treated twice with ether. The ether extracts were combined, washed with water, and dried. Removal of the ether and crystallization of the residue from acetone gave 10 mg. of crystalline material which melted at 66–68°. L-α-Octadecyl glycerol ether melts at 71–72°; the hexadecyl ether melts at 64° (18). 9.7 mg. of the hydrolysis product were dissolved in 2 ml. of absolute alcohol in a 5 ml. volumetric flask. 0.85 ml. of 0.2 M periodic acid was added and the solution was diluted to 5 ml. with absolute alcohol. The reaction mixture and a similarly prepared blank were allowed to stand in the dark at room temperature for 30 minutes. Then 1.0 ml. aliquots were removed and treated with excess arsenite solution and titrated with iodine in saturated bicarbonate in the usual way. The 1.0 ml. aliquot reduced 0.0052 mmole of periodate (theory = 0.0056 mmole). The degradation product thus reduced 0.93 mole of periodic acid per mole. In similar titrations α- and β-octadecyl glycerol ethers reduced, respectively, 1.02 and 0.03 moles of periodic acid per mole.

β-Octadecyl Glycerol Ether—This compound was prepared according to the directions of Davies, Heilbron, and Jones (17). 1,3-Benzylidene glycerol (1.6 gm.) was refluxed in 50 ml. of benzene with 207 mg. of potassium for 1 hour. Octadecyl iodide (2.6 gm.) was added, and refluxing was continued for a further 8 hours. The cooled mixture was poured into water and extracted with ether and the ether layer was washed three times with water, dried, and evaporated. The excess octadecyl alcohol was distilled off under reduced pressure. Crystallization of the residual 1,3-benzylidene glycerol-2-octadecyl ether from aqueous alcohol gave a waxy solid, m.p. 42–44°. This product was refluxed for 70 minutes in 35 ml. of 75 per cent aqueous alcohol containing 1 ml. of concentrated HCl. The solution was diluted with water, and the precipitated solid was crystallized several times from alcohol to furnish β-octadecyl glycerol ether, m.p. 60–61°. The material showed no melting point depression when admixed with an authentic sample of β-octadecyl glycerol ether, m.p. 60–61°, kindly supplied by Dr. P. E. Verkade.

β-Octadecyl Glycerol Ether α,α′-Diacetate—β-Octadecyl glycerol (52.5 mg.)

in 0.2 ml. of pyridine was heated with 1 ml. of acetic anhydride at 80–85° for 1 hour. The solution was allowed to stand for 12 hours at room temperature and then was evaporated under reduced pressure. The residual oil was dissolved in chloroform and the solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to a waxy solid (68.4 mg.). After repeated digestion with hot hexane and removal of the insoluble residue (6.6 mg., m.p. 100°) the filtrate was evaporated to a waxy solid. The infrared spectrum was consistent with the ether diacetate structure, the following bands being present: 2920 cm.$^{-1}$ and 3860 cm.$^{-1}$ (CH_3, CH_2, and CH stretch), 1740 cm.$^{-1}$ (ester C=O), 1470 cm.$^{-1}$ (—CH_2— deformation), 1375 (C—CH_3 deformation), 1238 cm.$^{-1}$ (C—O— stretch of acetate), and 1119 cm.$^{-1}$ (—CH—O—CH_2—). There was an additional very strong band at 1050 cm.$^{-1}$. Analysis of β-octadecyl glycerol ether α,α'-diacetate

$C_{25}H_{48}O_5$. Calculated, C 70.0, H 11.2; found, C 69.4, H 11.2

α-Octadecyl Glycerol Ether α,β-Diacetate—α-Octadecyl glycerol ether (batyl alcohol) (59.5 mg.) was acetylated in the above manner to furnish a waxy solid (63.7 mg.). Repeated crystallization from alcohol furnished soft crystals, m.p. 34–35° (Baer and Fischer (18) report m.p. 34–35°). The infrared absorption spectrum in chloroform solution displayed maxima at 2935 and 2870 cm.$^{-1}$ (CH_3, CH_2, and CH stretch), 1739 cm.$^{-1}$ (ester C=O), 1465 cm.$^{-1}$ (—CH_2— deformation), 1375 cm.$^{-1}$ (C—CH_3 deformation), 1230 cm.$^{-1}$ (C—O— stretch of acetate), and at 1116 cm.$^{-1}$ (—CH_2—O—CH_2). Additional bands were observed at 1048 cm.$^{-1}$ (strong) and 960 cm.$^{-1}$ (weak). There was no hydroxyl absorption at approximately 3500 cm.$^{-1}$.

Proton Magnetic Resonance Spectra—The proton magnetic resonance spectra were determined at 40 mc. rf. with a Varian model V4300B high resolution spectrometer fitted with a field-sensing stabilizer (Super Stabilizer). The compounds were measured as about 25 per cent solutions in chloroform or deuterochloroform. The chemical shifts expressed relative to water were actually determined relative to methylene chloride with a concentric tube cell (29) with methylene chloride in the outer compartment. Subtraction of 26 c.p.s. from shifts relative to methylene chloride was used in determining shifts in cycles per second relative to water. For the sake of clarity the spectra illustrated were run in the absence of methylene chloride. The actual shifts were measured on separate spectra in which the methylene chloride band was included.

SUMMARY

Alkaline hydrolysis of egg yolk phosphatides gives a crude sphingolipide fraction containing a ninhydrin-positive substance (ethanolamine-lipide).

The purified ethanolamine-lipide contains no sphingosine and has been shown to consist mainly of a phosphorylethanolamine derivative of batyl alcohol. The presence of similar glycerol ether phosphatides in other lipides is indicated by analytical data. Studies of the distribution of ether phosphatides are now in progress.

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ALLOXAN INACTIVATION OF COENZYME A AND THE PIGEON LIVER ACETYLATING ENZYME*

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(Received for publication, August 28, 1957)

In 1943, Dunn *et al.* (3) reported that the injection of alloxan into animals selectively destroyed the β cells of the islands of Langerhans in the pancreas. This discovery has led to much interest and speculation on the mechanism of action of this drug. Since alloxan has been shown to react with sulfhydryl groups, and since the prior injection of large doses of either glutathione or cysteine protects the animals from the effects of alloxan, Lazarow (4) has postulated that alloxan kills the β cells by inactivating certain essential sulfhydryl enzymes. Later studies (5, 6) have shown that, in addition to its ability to oxidize sulfhydryl groups, alloxan reacts with the sulfhydryl of glutathione to form a new compound which has an absorption spectrum maximum at 305 mμ and which is believed to be an addition product.

Many investigators have studied the reaction of alloxan with various enzymes, particularly those which contain essential sulfhydryl groups. However, in most cases the concentration of alloxan used was much greater than that which could be expected to occur within the cell after the injection of a diabetogenic dose of alloxan. If the intravenous diabetogenic dose of alloxan (40 mg. per kilo) (7) were uniformly distributed throughout the body water, calculated as 70 per cent of body weight, its maximal concentration within the cell would be less than 4×10^{-4} M. Since alloxan is rapidly destroyed at body temperature and pH (the half life being less than 1 minute (8)), it is unlikely that this compound could be concentrated to any great extent in specific cells such as the β cells within the short time before its destruction. This has been borne out by preliminary studies on the distribution of radioactive alloxan in the toadfish (9). After injection, the alloxan concentration in the isolated islet tissue never exceeded the level which would be expected if it were assumed to be uniformly distributed throughout body water, and at all times (2 to 30 minutes) after injection

* This investigation was supported by research grants No. A-824 and No. A-1659 from the Institute of Arthritis and Metabolic Diseases, Public Health Service. Preliminary reports have been published (1, 2).

the radioactivity of the islet tissue was less than 50 per cent of that of blood. It is therefore unlikely that the concentration of alloxan within the β cell after the injection of a diabetogenic dose of this compound would ever exceed 4×10^{-4} M.

Our interest in coenzyme A (CoA) as a possible site of action of alloxan stems from the work of Lipmann (10) and others, who have demonstrated its key role in cell metabolism. It is well known that CoA contains a sulfhydryl group which is essential for its activity; in fact, "active acetate" is a combination of acetate with the sulfhydryl group of CoA (11). One would certainly expect serious derangements of metabolism if alloxan interfered with the function of the sulfhydryl group of CoA.

Materials and Methods

The CoA activity was determined in the pigeon liver sulfanilamide-acetylating system of Kaplan and Lipmann (12), and for most of the experiments reported the pigeon enzyme was prepared as described by these authors. The CoA was obtained from the Pabst Laboratories. In this assay system the CoA solution was incubated with 0.25 ml. of the pigeon liver enzyme preparation for 2 hours at 37° in the presence of 3.94×10^{-4} M sulfanilamide, 2.46×10^{-2} M sodium acetate, 3.94×10^{-3} M adenosine triphosphate, 1.97×10^{-2} M sodium citrate, 8×10^{-2} M sodium bicarbonate, and 2×10^{-2} M cysteine hydrochloride.[1] At the end of this time the amount of sulfanilamide utilized was determined. In all experiments the disappearance (acetylation) of sulfanilamide was measured in both the presence and the absence of cysteine. The reaction was stopped by the addition of 4 ml. of 5 per cent trichloroacetic acid, and sulfanilamide was determined either by the method of Bratton and Marshall (13) or by a micromodification of this method. In the micromodification, a 50 μl. sample of the supernatant fluid was mixed with 100 μl. of 5 per cent trichloroacetic acid and 50 μl. of 0.1 per cent sodium nitrite. After 3 minutes, 50 μl. of 0.5 per cent ammonium sulfamate were added, and the mixture was again allowed to stand for 2 minutes. 50 μl. of *N*-(1-naphthyl)ethylenediamine dihydrochloride (1 mg. per ml.) were then added, and the optical density was read in a Beckman model DU spectrophotometer at 540 mμ.

With each new pigeon liver enzyme preparation, the amount of CoA required to catalyze 50 per cent of the maximal acetylation was determined in accordance with the method of Kaplan and Lipmann (12). This level (between 3 and 8 γ) was used in all subsequent tests. At this level, the

[1] It has been our experience that the cysteine hydrochloride should be added immediately before beginning the reaction. If it is added too early, the cysteine is oxidized to cystine, which is an effective inhibitor.

pigeon liver enzyme is present in excess and the amount of CoA therefore limits the reaction rate. For later experiments, when it became necessary to determine accurately the degree of inhibition of the liver enzyme by alloxan, the amounts of the components were adjusted so that the enzyme became limiting and the CoA was present in excess. Therefore, for this purpose each new pigeon liver preparation was also tested by measuring the degree of sulfanilamide acetylation in the presence of 20 γ of CoA and with varying amounts of the enzyme. The amount of sulfanilamide acetylated was proportional, within limits, to the amount of liver enzyme added, and the amount of liver to be used in the assay was selected from within this range.

In most of the experiments reported, anywhere between 40 and 60 per cent of the added sulfanilamide was acetylated by the control tubes during the 2 hour incubation period. In all experiments the amount of sulfanilamide acetylated was slightly smaller in the absence of cysteine than in its presence.

Results

Effect of Alloxan on Complete Acetylating System—When alloxan was added to the complete acetylating system to give a final concentration of 5×10^{-4} M (Table I), it inhibited acetylation by 53 to 60 per cent when the reaction was carried out in the absence of added cysteine. When cysteine was added, the inhibition was only about 15 per cent. When, however, the alloxan was previously incubated at room temperature for 60 minutes in phosphate buffer, pH 7.4 (the final pH of the solution being about 7.2), before its addition to the reaction mixture, there was insignificant inhibition of the acetylation reaction (7 per cent in the absence of cysteine and 5 per cent in its presence). Similar results were obtained when the alloxan was first incubated in sodium bicarbonate at pH 7.0 for 90 minutes. This observed loss in the ability of alloxan to inhibit the acetylation reaction after incubation at pH 7.0 to 7.2 is undoubtedly due to its decomposition, since it has been shown that alloxan rapidly decomposes in neutral or slightly alkaline solution (8). This observation therefore suggests that it is the alloxan *per se* and not a decomposition product of alloxan which inhibits the acetylation reaction.

Reaction of Alloxan with CoA—In the experiments shown in Table II, the alloxan was first incubated with CoA for 1 hour at room temperature in phosphate buffer, pH 7.4. Three different concentrations of alloxan and three different concentrations of CoA were used. At the end of this period the alloxan-CoA mixtures were added to the other components of the assay system and the acetylating activity was measured. In order to

TABLE I

Effect of Alloxan on Pigeon Liver Acetylating System

Each figure represents the average of six to thirteen determinations. During the preliminary incubation period the alloxan concentration was 1×10^{-2} M; when the alloxan was added to the acetylating test system, its final concentration was 5×10^{-4} M. CoA was limiting in the assay system.

Time of previous incubation of alloxan	Alloxan dissolved in			
	Phosphate buffer*		Sodium bicarbonate†	
	Cysteine in final reaction mixture			
	None	Added	None	Added
	Inhibition of acetylation			
min.	*per cent*	*per cent*	*per cent*	*per cent*
None	60	15	53	17
30	23	9	37	15
60	7	5	26	11
90	0	3	8	9

* The alloxan was dissolved in water and diluted to 1×10^{-2} M with Na_2HPO_4-KH_2PO_4 buffer, pH 7.4. The final phosphate concentration was 0.05 M, and the final pH about 7.2.

† The alloxan was dissolved in water and diluted to 1×10^{-2} M with $NaHCO_3$. The final bicarbonate concentration was 0.02 M, and the solution was adjusted to pH 7.0 with NaOH.

TABLE II

Reaction of Alloxan with Coenzyme A

Each figure represents the average of five determinations except as noted. The preliminary incubation was carried out in 0.05 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.4. In all cases the amount of CoA used in the final reaction mixture was 3.2 γ; therefore the CoA was limiting.

Alloxan concentration during preliminary incubation	Inhibition of acetylation					
	Cysteine omitted from assay system			Cysteine added to assay system		
	CoA concentration during preincubation					
	320 γ per ml.	160 γ per ml.	16 γ per ml.	320 γ per ml.	160 γ per ml.	16 γ per ml.
M	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2×10^{-3}	37	39	40	23	27	32
5×10^{-4}	25	29	17*	14	16	16
2.5×10^{-4}	10	6	11	5	3	4

* Average of four determinations.

eliminate any error due to spontaneous inactivation of CoA during the preincubation period, the control CoA samples were incubated for 1 hour in phosphate buffer, pH 7.4, in the absence of alloxan.[2]

It can be seen from Table II that 5×10^{-4} M alloxan inactivated between 17 and 29 per cent of the CoA, whereas, an alloxan concentration of 2.5×10^{-4} M produced insignificant inactivation. Since a 1 hour preincubation period destroys the ability of alloxan to inhibit the complete system (Table I), the observed alloxan effect must have been due to a direct reaction between alloxan and CoA.

Reaction of Alloxan and Pigeon Liver Acetylating Enzyme Complex—Table III shows the results of experiments in which alloxan was first incubated with the liver enzyme preparation for 1 hour at room temperature.

TABLE III

Reaction of Alloxan with Pigeon Liver Acetylating Enzyme Complex

Each figure represents the average of five determinations except as noted. CoA was limiting in the assay system.

Alloxan concentration during preliminary incubation	Inhibition	
	Cysteine omitted from assay system	Cysteine added to assay system
M	*per cent*	*per cent*
2×10^{-3}	88	31
5×10^{-4}	70	13
2×10^{-4}	47	2
1×10^{-4}	36*	1

* Average of four determinations.

In each case 1.35 ml. of the liver preparation were added to 0.15 ml. of an alloxan solution, and the degree of inactivation was determined by comparing the activity with control liver samples which had been incubated for 1 hour in the absence of alloxan.[3] In this set of experiments the assays were carried out under conditions whereby CoA was limiting.

The activity of the pigeon liver acetylating enzyme complex was markedly inhibited by low concentrations of alloxan and it would appear that the liver enzyme is much more sensitive to alloxan than is CoA. For example, although 2.5×10^{-4} M alloxan did not significantly inactivate CoA (Table II), a slightly lower alloxan concentration, 2×10^{-4} M, inhibited the activity of the pigeon liver enzyme complex by 47 per cent (Table III).

[2] Separate experiments have shown that there was no significant loss in CoA activity after a 1 hour incubation at room temperature in phosphate buffer.

[3] As in the case of CoA, incubation of the liver enzyme alone caused no inactivation.

Relative Sensitivity of CoA and Acetylating Enzyme Complex—In the earlier experiments (Tables II and III), the CoA and the liver enzyme had been previously incubated with alloxan under different conditions. In the present set of experiments the alloxan was incubated with the CoA or the liver acetylating enzyme under similar conditions; *i.e.*, in phosphate buffer at pH 7.0. In order to obtain the liver enzyme at the appropriate pH, the acetone powder was extracted with 0.05 M K_2HPO_4-KH_2PO_4, pH 7.6, instead of with 0.02 M $NaHCO_3$. The extract was frozen, thawed, and then centrifuged. The supernatant fluid was lyophilized, and the powder was redissolved in the same buffer just before use. The pH was adjusted

TABLE IV

Comparison of Effect of Alloxan on Coenzyme A and Pigeon Liver Acetylating Enzyme Complex

Each figure represents the average of four experiments.

Alloxan concentration during preliminary incubation	Inhibition			
	Alloxan incubated with CoA, 40 γ per ml.		Alloxan incubated with liver enzyme	
	Cysteine omitted from assay system	Cysteine added to assay system	Cysteine omitted from assay system	Cysteine added to assay system
M	*per cent*	*per cent*	*per cent*	*per cent*
2×10^{-3}	36	21		
1×10^{-3}	35	15		
5×10^{-4}	23	8	95	57
2.5×10^{-4}	7	7	93	42
1×10^{-4}			80	23
5×10^{-5}			60	16

to 7.0 with KOH, and the solution was diluted with water to the appropriate volume. The final phosphate concentration was 0.075 M. The CoA was dissolved in 0.1 M K_2HPO_4-KH_2PO_4, pH 7.1, and the pH of the final solution was found to be 7.0. Either 1.6 ml. of the CoA solution or 1.6 ml. of the liver enzyme were mixed with 0.4 ml. of four different concentrations of alloxan. The mixtures were incubated for 90 minutes and the pH of each mixture was determined at both the beginning and the end of the incubation period; only those experiments in which the pH was maintained between 7.0 and 7.1 are included. The degree of inactivation was determined by comparing the alloxan-treated samples with control mixtures incubated for 90 minutes in the absence of alloxan. The degree of inactivation of the CoA was measured in the assay system in which the CoA was limiting, and the degree of inactivation of the enzyme was measured in the assay system in which the liver enzyme was limiting.

The results (Table IV) show that the acetylation reaction was inhibited to a much greater extent when the liver enzyme complex was incubated with alloxan than was the case when the CoA was incubated with alloxan. Furthermore, it should be noted that in these experiments where the enzyme was limiting the observed inhibition was greater than that shown in Table III, where the CoA was limiting. This is to be expected since, in the experiments in Table III, a larger fraction of the enzyme could have been inactivated, but it would have escaped detection because the enzyme was present in excess.

The addition of cysteine to the final reaction mixture only partially reversed the inactivation of both the CoA and the liver enzyme complex (Table IV). Since the cysteine was added to the reaction mixture at a time when the alloxan was already destroyed, *i.e.* converted to alloxanic acid, this partial reactivation by cysteine is probably due to reduction of essential sulfhydryl groups of CoA and the liver enzyme which had presumably been oxidized by alloxan during the preliminary incubation period. On the other hand, when alloxan is added directly to the complete system in the presence of cysteine (Table I), the cysteine may also protect the enzyme system by reducing alloxan to dialuric acid, which is not diabetogenic (8). That part of the inactivation which is not reversed by cysteine is apparently due to another reaction, possibly the formation of an addition product between the alloxan and the liver enzyme or CoA.

DISCUSSION

As has been pointed out, many investigators have studied the effect of alloxan on different enzymes and enzyme systems. However, in most instances the concentration of alloxan required to produce significant inhibition was several times greater than the concentration of alloxan presumed to exist in the β cell after the injection of a diabetogenic dose of this compound. In the studies reported here, low concentrations of alloxan have been shown to inhibit both CoA and the acetylating enzyme complex. The alloxan sensitivity of this enzyme complex is greater than that previously reported for any other enzyme system; it is inhibited 80 per cent at 1×10^{-4} M and 60 per cent at 5×10^{-5} M. This inhibition is therefore very marked at concentrations well below the maximal alloxan concentration (4×10^{-4} M) which may exist in the β cell after the injection of a diabetogenic dose of alloxan.

The partial reversal of this inhibition by cysteine suggests the possibility that a generally occurring biochemical reaction may be selectively inhibited in those cells with a low sulfhydryl content; such a mechanism has been postulated (14) as a basis for the selectivity of alloxan for the β cells of the pancreas.

Although extensive inactivation of CoA could undoubtedly cause derangement of metabolism sufficient to kill a cell, CoA is not markedly inhibited at low alloxan concentrations. It is not known whether an inactivation of the acetylating enzyme complex could produce sufficient interference with metabolic activity to cause cell death. The pigeon liver acetylating enzyme complex consists of at least two components; one of these catalyzes the formation of acetyl CoA from acetate, adenosine triphosphate, and CoA; the other transfers the acetate from acetyl CoA to sulfanilamide (15). The former enzyme is probably widely distributed and important in metabolism, but the functional significance of the latter enzyme is unknown. We do not know which of these two enzymes is inactivated by alloxan. However, since the arylamine-acetylating enzyme contains an essential sulfhydryl group (16), it would seem to be a likely site. Further studies with more purified preparations (15) and with other CoA-requiring systems are in progress in an attempt to determine the exact site of alloxan inhibition.[4]

Although the high *in vitro* sensitivity of the acetylating enzyme system suggests that it may likewise be affected *in vivo*, more direct evidence must be obtained by studying islet tissue obtained from alloxan-injected animals. These studies will be carried out in the toadfish, in which the islet tissue is segregated into a discrete body and separated from the pancreatic acinar tissue (17, 18).

SUMMARY

The effect of alloxan on coenzyme A (CoA) and on the pigeon liver sulfanilamide-acetylating enzyme complex has been investigated. Alloxan, at a concentration of 5×10^{-4} M, produced a 23 per cent inactivation of CoA and 95 per cent inhibition of the liver acetylating enzyme complex. This acetylating enzyme was inhibited 60 per cent by 5×10^{-5} M alloxan, one-eighth of the maximal alloxan concentration which might exist in the cell after the injection of a diabetogenic dose of this compound. In all instances the inactivation was partly reversed by the addition of cysteine. The relationship of these results to the diabetogenic mechanism of alloxan is discussed.

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[4] Preliminary results obtained since this paper was submitted for publication indicate that both components of the acetylating enzyme complex may be equally sensitive to low concentrations of alloxan. The details will be published in a separate communication.

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THE OSMOTIC NATURE OF MITOCHONDRIAL SWELLING PRODUCED BY CARBON TETRACHLORIDE AND INORGANIC PHOSPHATE*

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(Received for publication, September 6, 1957)

By measurements from photographs, Tedeschi and Harris in a definitive work (1) showed that increases in mitochondrial volume can be followed by measurement of the optical density of the mitochondrial suspension. These experiments were performed with hypotonic solutions of non-penetrating substances and with isotonic solutions of penetrating substances. Mitochondrial swelling under these circumstances involves the movement of solvent caused by the osmotic gradient. As in studies of hemolysis (2), we may designate decreases in optical density under these conditions as "osmotic swelling." That a decrease in the optical density of a mitochondrial suspension actually involves an increase in mitochondrial water content has been shown by direct analysis by Price *et al.* (3) and has been confirmed in this laboratory. A quantitative correlation between the increase in volume of packed mitochondria and the decrease in optical density of mitochondria suspended in hypotonic sucrose has also been demonstrated.[1]

In contrast to the osmotic swelling of mitochondria is a decrease in optical density of mitochondrial suspensions produced by such agents as phosphate (4), thyroxine (5), triiodo- and tetraiodothyroacetic acids (6), and carbon tetrachloride (7). These agents produce swelling of mitochondria suspended in *isotonic* solutions of such non-electrolytes as sucrose and mannitol (8). The underlying mechanism which results in a decrease in optical density in an isotonic solution of a non-penetrating solute is not well understood (8, 9). This paper presents the results of a simple test system, which indicate that phosphate- and carbon tetrachloride-induced swelling of mitochondria in isotonic sucrose solution occurs as a result of a decrease in the mitochondrial membrane semipermeability.[2] Mito-

* Supported in part by grant No. A-329(C4) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

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[1] Malamed, S., and Recknagel, R. O., unpublished.

[2] Preliminary experiments have indicated that mitochondrial swelling induced by 3×10^{-6} M thyroxine, although delayed by hypertonic sucrose, may be brought

chondrial volume changes under these conditions are directly comparable to colloid osmotic hemolysis (10), which has been extensively discussed in the literature on red cell hemolysis as the dual mechanism for hemolysis (11, 12).

EXPERIMENTAL

Homogenization and Differential Centrifugation—Male rats (Holtzman Rat Company, Madison, Wisconsin), weighing from 200 to 300 gm. and fed *ad libitum*, were used. The animals were killed by cervical section and exsanguinated. The liver was removed and placed in ice-cold 0.30 M sucrose which was 0.002 M with respect to EDTA[3] at pH 7.4. This medium was used for all the homogenization and differential centrifugation steps, except for the final suspension of the mitochondria which was in 0.30 M sucrose. The temperature during preparation of mitochondria was 0–2°. An all Lucite homogenizer of the Potter-Elvehjem type was used. 10 per cent homogenates of whole rat liver were centrifuged (International table model centrifuge, rotor No. 215) for 15 minutes at 600 × *g* to remove whole cells and nuclei. The supernatant fraction was centrifuged (Spinco preparative ultracentrifuge, model L, rotor No. sw-25.1) for 12 minutes at 10,000 × *g* to sediment the mitochondria. Upon resuspension and resedimentation, a fluffy layer (13) which appeared above the mitochondria was removed.

Analytical Methods—Swelling of mitochondria was followed by decrease in the optical density of the suspension at 520 mμ in a Coleman spectrophotometer, model 6A. The starting point of the time studies was the final addition of 0.2 ml. of a 50 per cent mitochondrial suspension to 2.8 ml. of a suspension medium which was routinely 0.002 M with respect to Tris buffer at pH 7.4 and which contained other additive substances as indicated below. The temperature of the mitochondrial suspension was 0–2° when added to the colorimeter tubes; the other components were at room temperature. Tris and ATP were products of the Sigma Chemical Company and erythritol was obtained from the Nutritional Biochemicals Corporation. Other substances used were of analytical reagent grade. Human red cells, prepared according to Ponder (12), were used for the hemolytic studies.

Results

Carbon Tetrachloride-Induced Swelling—As shown in Fig. 1, the optical density of a suspension of mitochondria in isotonic sucrose solution de-

about by changes other than, or in addition to, altered mitochondrial membrane permeability.

[3] The abbreviations used are EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate; AMP, adenosine monophosphate.

creases with time when carbon tetrachloride is present. The degree of swelling is greater with higher levels of saturation of the medium by carbon tetrachloride. The various percentage saturations of carbon tetrachloride were obtained by appropriate dilution of a stock solution with 0.30 M sucrose. The stock solution was made by shaking a mixture of 1 volume of carbon tetrachloride with 5 volumes of 0.30 M isotonic sucrose solution for 30 minutes. No carbon tetrachloride phase as such was present in the final reaction medium. Carbon tetrachloride induces mitochondrial swelling when other solutes are substituted for sucrose. Table I shows the re-

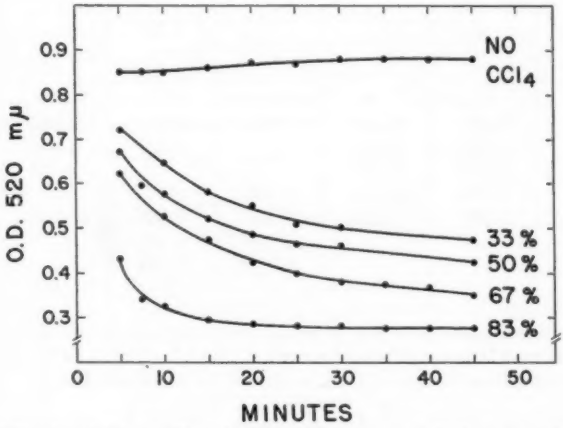

FIG. 1. Mitochondrial swelling in isotonic sucrose solution containing CCl_4. Conditions: 2.6 ml. of 0.30 M sucrose containing CCl_4 as indicated; 0.2 ml. of 0.03 M Tris buffer, pH 7.4; 0.2 ml. of a 50 per cent suspension of mitochondria in 0.30 M sucrose. The figures represent the percentage of saturation with respect to carbon tetrachloride. The concentration of carbon tetrachloride at 100 per cent saturation is estimated to be 0.005 M.

sults with isosmolar KCl, NaCl, and Na_2SO_4. In the case of Na_2SO_4, although swelling occurred in the absence of carbon tetrachloride, it was enhanced when carbon tetrachloride was present. The observation of Tapley (14) that mitochondria swell slowly in isotonic sucrose solution if they have been previously washed free from such protective agents as ATP and EDTA has been confirmed in this laboratory. The swelling of mitochondria prepared in this manner is greatly accelerated by carbon tetrachloride.

Osmotic Nature of Mitochondrial Swelling in Isotonic Sucrose Solutions—It is well known that red cells will hemolyze when suspended in isotonic glycerol solution. Swelling begins immediately when the glycerol penetrates the red cell membrane and the intracellular osmotic pressure tends to

rise. As has been pointed out by Ponder ((12), p. 243, footnote 60) and Davson ((15), p. 150), hemolysis of red cells produced by the penetration into the red cell of an external solute should be delayed if the concentration of the

TABLE I

Mitochondrial Swelling in Isotonic Solutions of Electrolytes Saturated with Carbon Tetrachloride

The optical density was at 520 mμ.

Time	KCl	KCl + CCl_4	NaCl	NaCl + CCl_4	Na_2SO_4	Na_2SO_4 + CCl_4
sec.						
20				0.700		
30						0.650
45				0.590		
min.						
1	0.875	0.560	0.875	0.550	0.850	0.610
2	0.860	0.490		0.520	0.750	0.570
3	0.860	0.480			0.720	
4	0.860	0.470	0.860			
5	0.860	0.460	0.860	0.500	0.650	0.530
6			0.860	0.470		
8			0.860	0.470		
10					0.600	0.500

The conditions were as in Fig. 1, except that 0.15 M KCl, 0.15 M NaCl, or 0.1 M Na_2SO_4 was used in place of isotonic sucrose. 2.6 ml. aliquots of the salt solutions previously equilibrated against CCl_4 were used to induce swelling.

TABLE II

Delay of Red Cell Hemolysis in Hypertonic Solutions of Glycerol

Final concentration of glycerol	Time to reach optical density of 0.150
M	*min.*
0.26	0.75
0.53	3.00
0.79	5.25

Conditions: 0.4 ml. of a standard red cell suspension was added to 3.0 ml. of glycerol solution at the appropriate molarity. For other conditions, see the text.

external solute is elevated. The delay in hemolysis would be expected since a finite time would be required for the osmotic pressure of the intracellular compartment first to equal and then to exceed the external osmotic pressure. The delay for red cell hemolysis in hypertonic solutions of glycerol is shown in Table II. Hemolysis was followed at 15 second intervals at 700 mμ. The time required to reach a fixed end point (0.150 optical density unit) was determined from the resulting hemolysis rate

curves. The data clearly reveal the marked delay in hemolysis time with increasing external glycerol concentration.

TABLE III

Delay of Mitochondrial Swelling in Hypertonic Solutions of Erythritol

Final concentration of erythritol	Time to reach optical density of 0.450
M	min.
0.26	8.0
0.39	11.0
0.52	12.0
0.78	14.5
1.04	18.0

The conditions were as in Fig. 1, except that 2.6 ml. of the appropriate erythritol solution were used in place of isotonic sucrose.

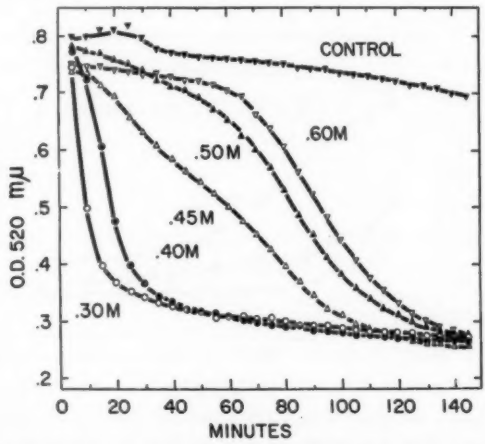

FIG. 2. Delay in phosphate-induced mitochondrial swelling in hypertonic sucrose. The conditions were as in Fig. 1, except that 2.6 ml. of the appropriate hypertonic sucrose solution were used. The final concentration of inorganic phosphate was 0.02 M (potassium phosphate buffer at pH 7.4).

As in the case of the red cell, mitochondria swell immediately (1) when placed in isotonic solutions of a penetrating solute. The expected delay in swelling when the mitochondria are suspended in hypertonic solutions of a penetrating solute is shown in Table III. Mitochondrial swelling was followed at 1 minute intervals at 520 mμ. The time required to reach a fixed end point (0.450 optical density unit) was determined from the resulting mitochondrial swelling rate curves. Erythritol was chosen for

convenience since the swelling rate in glycerol solutions is too rapid for accurate measurement with the instrument employed. The data of Table III clearly reveal the marked delay in mitochondrial swelling time

TABLE IV

Mitochondrial Swelling in Sucrose Solutions Saturated with CCl_4

Sucrose molarity	Optical density at 520 mμ	
	3 min.	35 min.
0.30 (no CCl_4)	0.810	0.830
0.30	0.530	0.285
0.40	0.585	0.279
0.50	0.590	0.259
0.60	0.640	0.262
0.70	0.670	0.262

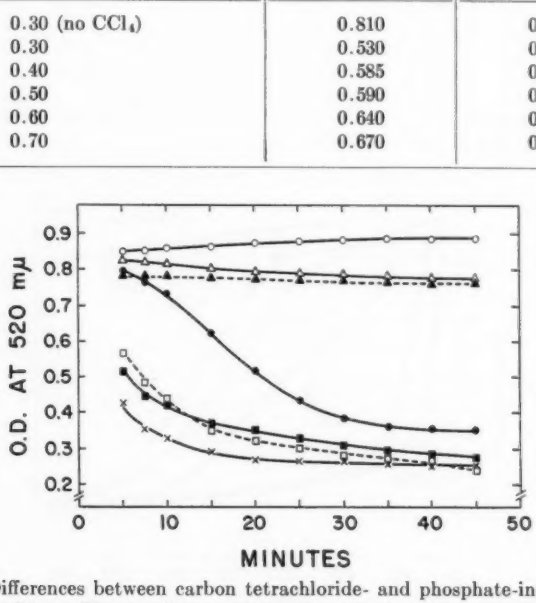

FIG. 3. Differences between carbon tetrachloride- and phosphate-induced mitochondrial swelling. The conditions were as in Fig. 1. The concentrations used were carbon tetrachloride, 83 per cent saturated; 0.02 M potassium phosphate buffer, pH 7.4; 0.004 M ATP; 0.002 M EDTA. ○, control; △, potassium phosphate plus ATP; ●, potassium phosphate; ▲, potassium phosphate plus EDTA; □, CCl_4 plus EDTA; ■, CCl_4 plus ATP; ×, CCl_4.

with increasing external erythritol concentration. This result was predictable from the work of Tedeschi and Harris (1).

This type of experiment was applied to the mitochondrial swelling produced by phosphate and carbon tetrachloride. Clearly, if the mitochondrial swelling produced in isotonic sucrose solutions containing these agents is caused by a decrease in the mitochondrial permeability to sucrose, a delay in the decrease in optical density would be expected if the external

sucrose concentration were elevated. Typical data for phosphate-induced mitochondrial swelling are shown in Fig. 2. It may be noted that the final end point reached is identical for all sucrose concentrations used, as would be predicted by the colloid osmotic theory. The data (Table IV) show the effect of increased hypertonicity in producing an initial delay (at 3 minutes) in carbon tetrachloride-induced swelling, although the final end points (at 35 minutes) are the same. These data on phosphate- and carbon tetrachloride-induced swelling clearly reveal the marked delay as the concentration of the external sucrose solution is elevated.

Comparison of Carbon Tetrachloride- and Phosphate-Induced Swelling—Although an osmotic mechanism induces both carbon tetrachloride- and phosphate-induced swelling, there are differences in the actions of these agents. Fig. 3 shows the relative lack of protection afforded by ATP or EDTA against swelling in 0.30 M sucrose solution saturated with carbon tetrachloride, in contrast to the complete effectiveness of both substances in protecting the mitochondria against phosphate-induced swelling. In addition, the onset of phosphate-induced swelling is slow in comparison to that with carbon tetrachloride-induced swelling. These differences suggest different modes of action on the mitochondrial membrane, although the final result, the breakdown of mitochondrial membrane semipermeability, appears to be the same in both cases.

DISCUSSION

Fonnesu and Davies (9) state that "Mn^{2+} or Mg^{2+} ions plus AMP can largely protect non-respiring liver mitochondria from swelling even in solutions as dilute as 40 mM sucrose." If one assumes the intramitochondrial concentration to be 0.3 osmolar, the osmotic gradient with a 40 mM external sucrose concentration would be in excess of 7 atmospheres. According to Werkheiser and Bartley (16), rat liver mitochondria are permeable to sucrose to the extent of 60 per cent of the intramitochondrial water, and are possibly even somewhat permeable to molecules as large as inulin. These views contradict the widely held idea that the mitochondria behave as osmometers and that the mitochondrial membrane is a relatively impermeable structure. Compelling evidence in favor of the latter view is the work of Tedeschi and Harris (1), who showed that the rat liver mitochondrial membrane exhibits permeability characteristics remarkably similar to cell membranes which in general are relatively impermeable structures. Schneider *et al.* (17) showed that virtually all of the endogenous liver citrate, either normally or when elevated 7-fold in fluoroacetate poisoning, could be recovered in the isolated mitochondrial fraction. The capacity of isolated mitochondria to retain soluble enzymes, coenzymes, nucleotides, and ions has been widely observed. The osmotic behavior of

the mitochondria under the experimental conditions described in this publication is also consistent with the view that the mitochondrial membrane is normally impermeable to sucrose and that the osmotic behavior of mitochondria follows the expectations of classical osmotic pressure theory. The significance of the work reported here is that it supports a theoretical approach which envisages two types of mitochondrial swelling, *viz.* simple osmotic swelling and colloid osmotic swelling. The first of these involves either the osmotic adjustment to hypotonic conditions or the swelling observed in isotonic solutions of penetrating substances. The second type of mitochondrial swelling, observed in isotonic solutions of normally non-penetrating substances, occurs as a result of a decrease in the mitochondrial membrane permeability. If the impermeability of the mitochondrial membrane to protein is at least partly maintained, excess intramitochondrial osmotic pressure due to electrostatically bound intramitochondrial electrolyte would result in mitochondrial swelling, even if some loss of osmotically active material from the mitochondria did occur. Swelling under these conditions would then be due to the osmotic imbalance conditioned by the Donnan equilibrium.

Colloid osmotic swelling is markedly influenced by adenine nucleotides and divalent metals, as the work of Fonnesu and Davies (9) and others clearly indicates. However, the conclusion (9) that these agents protect against swelling should in our view be limited to colloid osmotic swelling and not to the rapid initial adjustment of mitochondria to hypotonic conditions. This does not preclude the possibility that adenine nucleotides or other agents may not affect the *rate* of adjustment of mitochondria to hypotonic media.

Werkheiser and Bartley (16) centrifuged mitochondria through a column of silicone dissolved in an organic solvent. In view of the rapidity of the changes induced by dilute aqueous carbon tetrachloride,[4] it appears to us that addition of a mitochondrial suspension above a non-aqueous phase could easily lead to significant penetration of the mitochondria by sucrose or other normally non-penetrable substances before and during the centrifugation procedure used by these workers.

SUMMARY

A simple test system is presented which confirms that an expected delay in the hemolysis of red cells in hypertonic glycerol and in the swelling of

[4] Unpublished experimental work in this laboratory has indicated that the effectiveness of aqueous solutions of fat solvents in inducing a characteristic ATPase transformation (18), indicative of mitochondrial damage, in isolated normal rat liver mitochondria is inversely proportional to the water solubility of the fat solvent. The effectiveness of a series of organic solvents was thus found to be $CCl_4 = C_6H_6 > CHCl_3 > CH_2Cl_2 > CH_3CH_2OCH_2CH_3$.

mitochondria in hypertonic erythritol, predictable from classical osmotic pressure theory, is observed experimentally.

Application of this test to the mitochondrial swelling observed in isotonic sucrose solution in the presence of phosphate and carbon tetrachloride indicates that mitochondrial swelling produced by these agents is caused by a primary increase in the mitochondrial membrane permeability, accompanied by movement of solvent into the mitochondria.

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INFLUENCE OF POLYPHENOLS AND POTATO COMPONENTS ON POTATO PHOSPHORYLASE

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(Received for publication, November 4, 1957)

Results reported previously demonstrated that inorganic phosphate in ethanolic extracts of potatoes inhibited the formation of long chain amyloses by phosphorylase without influencing the rate of liberation of inorganic phosphate from glucose 1-phosphate (1, 2). It was also learned that, in deionized extracts, liberation of inorganic phosphate in a phosphorylase assay system containing suboptimal concentrations of a starch primer was accelerated. This finding implied the presence in the original extract of true phosphorylase inhibitors capable of counteracting the effect of the activators (3). The present paper includes a report on the phosphorylase-affecting properties of fractions of the extract prepared in an attempt to isolate the inhibitory fraction. Consideration of these properties has led to the tentative conclusion that the inhibitory activity is associated with polyphenolic acid (or acids). Also included are the results on the inhibition of phosphorylase by substances derived from anion exchange resins and by polyphenols and on the activation of phosphorylase by potato fractions and by a heat-stable, amylase-resistant component of human saliva.

EXPERIMENTAL

The preparation, assay, and definition of units of enzyme have been previously described (2). To test the effect of each fraction described in Tables I and II, an aliquot of 0.5 ml. at pH 6.0 was added to the standard incubation mixture of 1 ml. containing 2.5 units of phosphorylase. All assays were carried out in duplicate in comparison with zero time controls. The effect of the addition substance was always compared with that of a control without an addition compound. Also, the possible effect of the addition compounds on the determination of inorganic phosphate was ascertained by recovery studies. In no instance did any of the addition substances used here interfere with the determination of inorganic phosphate. When the potato fractions were manipulated to determine the effects of various treatments on the inhibitory activity, the volumes were adjusted to that of the original fraction after pH adjustment to 6.0.

Results

Inhibition by Polyphenols—Fig. 1 shows that chlorogenic acid inhibits the activity of potato phosphorylase. Table I shows that caffeic acid, catechol, and epinephrine also inhibit enzyme activity, although these substances are not as potent as chlorogenic acid. No inhibition was observed in the presence of tyrosine or of dihydroxyphenylalanine; the latter exerts an activating effect which is possibly significant. Bisulfite and ascorbic acid caused a slight inhibition.

Since caffeic acid exerts a more powerful inhibitory effect than quinic acid, it is probable that the inhibitory action of chlorogenic acid, which is the caffeyl ester of quinic acid, is primarily associated with the caffeyl

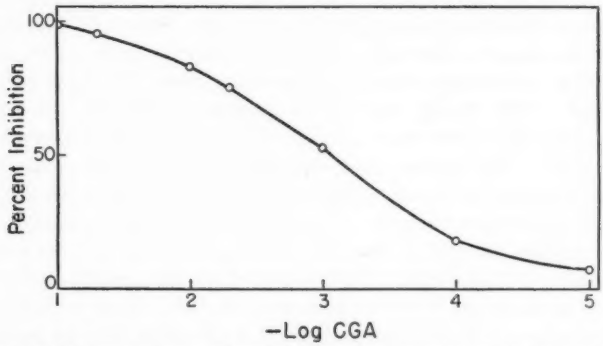

FIG. 1. Inhibition of potato phosphorylase as a function of the logarithm of the reciprocal of chlorogenic acid concentration (molar) in the enzyme reaction mixture.

moiety. The lack of inhibition by dihydroxyphenylalanine coupled with the observation that certain reducing substances are also slightly inhibitory would tend to eliminate any explanation based on the oxidation of essential enzyme sulfhydryl groups via orthoquinones formed from the corresponding phenols. Quastel (4) showed that such an explanation is valid for the inhibition of urease by polyhydric phenols. Fischer and Hilpert (5) found that phloretin, the aglycone of phlorizin, a polyphenolic glucoside related to the anthocyanins, is a powerful inhibitor of potato phosphorylase. As is well known, phlorizin inhibits animal but not plant phosphorylase (5, 6).

Purification of Inhibitor

Step 1—1 kilo of peeled, diced potatoes (Russet Burbank variety) was extracted with 5 liters of hot 80 per cent ethanol. After blending, filtering, and reextraction of the residue, the ethanol was removed from the combined extracts by evaporation *in vacuo* at room temperature, and water

was added to the residue to 50 ml. (Fraction I, Table II). In agreement with previous observations, no inhibition by Fraction I was observed.

TABLE I

Inhibitors of Potato Phosphorylase

Substance	Concentration	Per cent inhibition	I^{M*}_{CGA}
	M		
Chlorogenic acid	10^{-5}–10^{-1}	7–100	1.00
Caffeic acid	5×10^{-2}	85	0.30
Quinic "	5×10^{-2}	10	<0.01
Catechol	5×10^{-2}	57	0.03
Epinephrine	1×10^{-2}	65	0.30
Tyrosine	2.5×10^{-3}	0	0.00
Dihydroxyphenylalanine	5×10^{-2}	−17	
"	1×10^{-2}	1	0.00
"	1×10^{-3}	0	0.00
Ascorbic acid	5×10^{-2}	16	0.02
Sodium bisulfite	2×10^{-2}	45	0.03

* I^{M}_{CGA} = ratio of molar concentration of chlorogenic acid corresponding to the observed inhibition to molar concentration of test substance in the reaction mixture.

TABLE II

Preparation of Potato Phosphorylase Inhibitor

Fraction No.	Treatment	$I^{mg.*}_{CGA}$	Total inhibitory activity†
I	80% ethanol	0.000	0
II	Lipide removal	0.000	0
III	90% ethanol	0.006–0.010	50–70
IV	Activated carbon	0.014–0.010	84–95
V	Anion exchange	4 −8	
VI	Absolute ethanol	1.1 −1.7	72–99

* $I^{mg.}_{CGA}$ = chlorogenic acid inhibitory index = ratio of mg. of chlorogenic acid corresponding to the observed inhibition in the presence of the fraction to mg. of fraction used per assay.

† Total inhibitory activity = $I^{mg.}_{CGA} \times$ mg. of fraction.

Step 2—The lipides were extracted with three 50 ml. aliquots of petroleum ether, and the volume of the aqueous phase was adjusted to 100 ml. (Fraction II). Both the lipide and the lipide-free fractions were devoid of inhibitory activity.

Step 3—Absolute ethanol was added to Fraction II so that the final volume of the suspension was 1 liter. After remaining at 4° for 17 to 20 hours, the suspension was filtered by gravity. The filtrate was then evapo-

rated to dryness, and the residue was taken up in 25 ml. of water. Insoluble residue, which was shown to be tyrosine, was removed by centrifugation to yield 25 ml. of supernatant solution (Fraction III). The precipitate from the treatment with ethanol exhibited phosphorylase-priming activity. Fractionation at room temperature was not effective in separating inhibitor from activator. Additional primer activity could be separated from Fraction II by precipitation with 0.4 saturated ammonium sulfate.

Step 4—To Fraction III were added 2.5 gm. of activated carbon (Norit A). After 30 minutes, the carbon was filtered off on a layer of Celite. After washing with 200 ml. of water, the filtrates were combined and diluted to 250 ml. (Fraction IV).

Almost all of the brown color was removed by Step 4. Prolonged exposure to carbon resulted in a progressive loss of inhibitory activity from solution. The apparent increase in total inhibitory activity (Table II) indicates that residual primer was removed by this treatment.

Attempts to remove this residual primer activity via hydrolysis with salivary amylase (7, 8) resulted in the finding that saliva contains a heat-stable activator of phosphorylase. As little as 0.02 ml. of boiled saliva per ml. of enzyme reaction mixture more than doubled the rate of liberation of inorganic phosphate.

Step 5—Fraction IV was placed on a column (3 × 5 cm.) of Dowex 1 acetate anion exchange resin prepared from Dowex 1-X10 chloride, 200 to 400 mesh (9). After the bulk of the volume was taken up by the column, 200 ml. portions of the following were passed through at the rate of 1.5 ml. per minute under positive nitrogen pressure: water, 0.33 N acetic acid, and 1 N acetic acid. The acetic acid was removed *in vacuo* at 40° from the effluent coming through with 1 N acetic acid (Fraction V).

Part of the inhibitory activity of this fraction was due to the presence of an artifact derived from the ion exchange resin. Repeated washings with 3 N acetic acid and the use of a three cycle regenerated resin did not eliminate the appearance of inhibitory activity in appropriate blanks. This resin-derived inhibitor is a green solid insoluble in carbon tetrachloride, acetone, and alkali, but soluble in dilute acid and in slightly acidified absolute ethanol. Ultraviolet absorption spectroscopy revealed a distinct peak with a maximum at 290 mμ. The inhibitory activity of this fraction corresponded to a chlorogenic acid inhibitory index equal to 2.5 (see Table II).

Step 6—Fraction V was taken up in 50 ml. of absolute ethanol, and the resulting suspension was centrifuged at 100,000 × *g*. The residue was resuspended in 25 ml. of absolute ethanol and recentrifuged, and the residues were dried *in vacuo* (Fraction VI).

A summary of yields and inhibitory action of the fractions is shown in Table II.

Observations Relating to Polyphenolic Acid Nature of Inhibitor—The properties of the inhibitor preparation as revealed by the purification procedure are in accord with the assumption that we are dealing with one or more polyphenolic acids. The following observations further substantiate this premise: (*a*) Preparations of inhibitor from potato peel were found to be much more potent than those from peeled tubers. Chlorogenic acid and other polyphenols are concentrated in the area under the peel (10–12). (*b*) Inhibitor preparations turn orange in the presence of acidified ammonium molybdate, indicative of the presence of polyphenols (13). (*c*) The inhibitory activity is adsorbed on anion but not on cation exchange resins, indicative of the acidic nature of the inhibitor. (*d*) Inhibitory activity is not associated with phosphate ester fractions of the potato. (*e*) Neutralization of slightly alkaline solutions of inhibitor resulted in a drift towards alkalinity, indicative of the presence of a lactonizing group. Many polyphenolic acids can undergo lactonization. (*f*) Dried neutralized preparations turned brown and lost inhibitory activity. (*g*) Fresh preparations exhibited a diffuse maximal absorption between 295 and 320 mμ, which upon subsequent deterioration was replaced by general absorption in the ultraviolet region, the absorbancy increasing with decreasing wave length. This behavior is quite typical of solutions of neutralized chlorogenic acid. (*h*) The inhibitory activity of the purest preparation was of the same order of magnitude as that of chlorogenic acid (see Fraction VI, Table II).

DISCUSSION

The results presented herein demonstrate that potatoes contain both activator and inhibitor of potato phosphorylase. Considerations of the properties of purified inhibitor fraction suggest that the inhibitory activity is associated with fairly strong acids which are polyphenolic in nature. Based on the data of Hunter *et al.* (12) on the total polyphenol content and on the concentration of phosphorylase in the potato tuber (14), one can calculate that there are approximately 5 μeq. of polyphenol per 2.5 units of phosphorylase in a tuber. The data of Fig. 1 show that this concentration will inhibit 2.5 units to the extent of 75 per cent. In the peel area there is probably enough polyphenol to suppress completely phosphorylase action.

Inhibition by phenols may be related to the presence of pyridoxal phosphate, which was recently reported to be present in pure preparations of crystalline muscle phosphorylase (15, 16). Braunstein (17) has stated that many polyphenols and quinones inhibit transaminases in concentrations similar to those effective in inhibiting potato phosphorylase. Recently Horvath (18) has reported that thyroxine also inhibits transaminases. Weinbach (19) found that phosphorylation at the glycolytic level is inhibited by concentrations of pentachlorophenol of the same order

of magnitude as that of chlorogenic acid effective in inhibiting potato phosphorylase.

The rather unexpected result that heated saliva can activate potato phosphorylase indicates that an α-amylase-resistant, thermostable component of saliva can function as a primer of amylose synthesis or can potentiate the activity of a starch primer added to the enzyme assay system.

The finding of potent enzyme inhibitors associated with ion exchange resins may serve as a note of caution in interpretation of enzymic effects of substances separated by this means. Hurlbert *et al.* (20) only partially eliminated colored and ultraviolet-absorbing material from Dowex 1 chloride columns by treatment with high concentrations of formic acid.

SUMMARY

It has been demonstrated that ethanolic extracts of potato contain both activators and inhibitors of potato phosphorylase. A procedure has been developed for the separation of these effects and for the enrichment of the inhibitory fractions. The properties and distribution of the inhibitory fractions indicate that most of the inhibitory activity is associated with polyphenolic acids present in the tuber. It has been found that certain polyphenols do inhibit activity of phosphorylase, the most potent of those tested being chlorogenic acid, one of the few polyphenols of potatoes so far identified. These studies have also led to the finding that human saliva is capable of activating phosphorylase after the amylase therein is destroyed by heat. Finally it has been demonstrated that certain anion exchange resins shed potent artifactual potato phosphorylase inhibitors when eluted with acid. The significance of these findings with respect to the mechanisms of action of these inhibitors and their possible metabolic role in polysaccharide synthesis are discussed.

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STUDIES IN STEROID METABOLISM

VII. CHARACTERIZATION OF BIOSYNTHETIC TESTOSTERONE GLUCOSIDURONIC ACID AS METHYL-[(TESTOSTERONE-17β)-2,3,4-TRI-*O*-ACETYL-β-D-GLUCOPYRANOSID]-URONATE*

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(Received for publication, December 10, 1957)

Recently three independent groups have shown the existence of testosterone glucosiduronic acid by the incubation of testosterone with rat liver (1) or with human prostatic tissue (2) and after the administration of large amounts of testosterone to humans (3). The evidence for identification rested mainly on the behavior on paper and the spectral characteristics of the steroid liberated by hydrolysis with β-glucuronidase. That the substance is a monoglucuronide was clear from data which showed equimolar amounts of testosterone and glucuronic acid (1) in the conjugate, and hydrolyzability of the latter by β-glucuronidase indicated a β-glucosidic linkage (1–3). The glucuronic acid radical was assigned to position 17 because the absorptions in the ultraviolet region of the conjugate and free testosterone were similar. This would exclude ring A and thus leave only ring D as the site of conjugation.

The location of the glucuronic acid radical in testosterone glucosiduronic acid has now been questioned since (synthetic) androstenedione-3-enol glucosiduronic acid (4) possesses an ultraviolet spectrum indistinguishable from that of biosynthetic testosterone glucosiduronic acid. The possible existence of enol glucuronosides in nature was considered in 1953 (5). Quite certainly, the same ultraviolet spectrum could be expected for testosterone-3-enol glucosiduronic acid. Thus, all the previous data (1–3) could be explained as well by the existence of testosterone-3-enol-β-D-glucosiduronic acid rather than by the presence of the glucuronic acid conjugate at position 17 of testosterone.

There is a need, therefore, to characterize chemically the biosynthetic testosterone glucosiduronic acid, at least to the extent which will provide

* These studies have been carried out with the assistance of research grants from the American Cancer Society, the National Institutes of Health, and Aids for Cancer Research (Boston).

an accurate picture of its structure. This has been done by comparing the infrared spectrum of authentic methyl-[(testosterone-17β)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate with that of the triacetyl methyl ester of testosterone glucosiduronic acid isolated from rat liver incubation mixtures. Proof of structure of tetrahydrocortisone glucosiduronic acid by comparing its triacetyl methyl ester with the authentic derivative was accomplished earlier in similar fashion by Schneider *et al.* (6).

EXPERIMENTAL

The product of many metabolic experiments carried out with rat liver slices and testosterone (1, 7) was collected as the ethyl acetate extract. The pooled residues (approximately 5 mg.) after evaporation of the solvent were esterified with methanol by the addition of 5.0 ml. of methanolic HCl (0.075 M). The solvent was removed by evaporation below 10°. To this residue, 3.0 ml. of chilled pyridine and 3.0 ml. of acetic anhydride were added in the cold. The reaction was allowed to take place at 0° for 20 minutes and then at room temperature for 24 hours. The volume of the mixture was reduced by evaporation *in vacuo* below 40° and this material was poured into 70 ml. of ice water with stirring. An oil separated out which gradually solidified when left for several weeks in the cold. This was purified by dissolving it in 95 per cent ethanol and by treating the ethanolic solution with charcoal. The mixture was filtered and water was added to the filtrate until turbidity appeared. This turbid mixture was left in the icebox and yielded an amorphous solid.

The amorphous material was recrystallized twice from 50 per cent methanol, yielding a tan-colored semicrystalline residue. This was dissolved in methanol, decolorized with a little Norit, and water was added until clouding occurred. After refrigeration for 96 hours, a white precipitate was obtained. The material was redissolved in methanol and one-twentieth of the sample was diluted to 10 ml. with methanol. This sample then was analyzed in a Beckman model DK2 ultraviolet spectrophotometer in the region from 340 to 220 mμ (Fig. 1). A maximum was found at 240 mμ corresponding in intensity to 130 γ of methyl-[(testosterone-17β)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate (total 2.6 mg.). The solution was then evaporated under a stream of nitrogen and one-fifth of the sample was analyzed in a Perkin-Elmer model 21 infrared spectrophotometer as a melt and was compared to a spectrum of methyl-[(testosterone-17β)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate (Fig. 2).

This reference compound was considered to be authentic for the following reasons: The route of synthesis was that described by Shapiro (8) and the product had the expected properties. Thus, the carbon and hydrogen analyses calculated for $C_{32}H_{44}O_{11} \cdot C_2H_5OH$ are C 62.73 per cent, H 7.74 per

cent; found, C 62.50 per cent, H 7.51 per cent. The melting point reported (8) was 186–189° and the prepared substance melted from 188.2–188.5°. Moreover, deacetylation with barium hydroxide, followed by

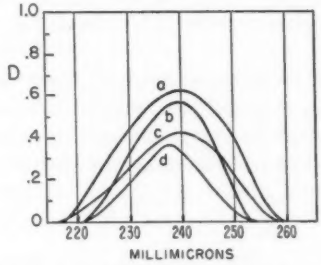

FIG. 1. Ultraviolet spectra of Curve a, synthetic methyl-[(testosterone-17β)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate; Curve b, acetylated and esterified product obtained from liver-testosterone incubation mixtures; Curve c, androstenedione-3-enol β-D-glucosiduronic acid; Curve d, methyl-[androstenedione-3-enol-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate. All spectra were recorded on a Beckman model DK2 spectrophotometer in methanol solution.

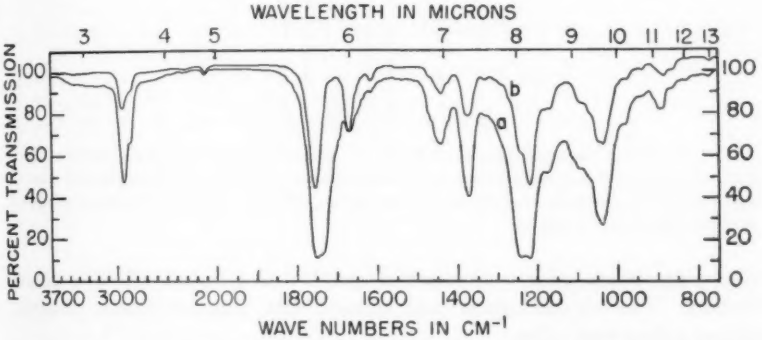

FIG. 2. Infrared spectra of (Curve a) esterified and acetylated product from liver-testosterone incubations and (Curve b) synthetic methyl-[(testosterone-17β)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate. Spectra were recorded as a melt in a Perkin-Elmer model 21 infrared spectrophotometer.

incubation with β-glucuronidase, quantitatively yielded testosterone. Further evidence of the structure of this substance was obtained from a study of the infrared spectrum (Fig. 2, Curve b). This showed the expected high in density absorptions for the four ester groups of the sugar moiety near 1750 cm.$^{-1}$ and near 1220 cm.$^{-1}$ and 1040 cm.$^{-1}$. The absence of hydroxyl stretching vibration bands confirms the position of the glucosidic

linkage at C-17, as do the ultraviolet spectrum and the blue Zimmermann color given by the product.

As can be seen in Figs. 1 and 2 from the coincidence of the absorption peaks both in the ultraviolet and infrared regions, the material isolated from the incubation *in vitro* appears to be identical in all respects with the synthetic material.

In Fig. 3 are shown the results of the paper chromatographic analysis of the conjugate, including a mixed chromatogram with authentic methyl-[(testosterone-17β)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate which shows no separation of the two substances, thus further verifying their iden-

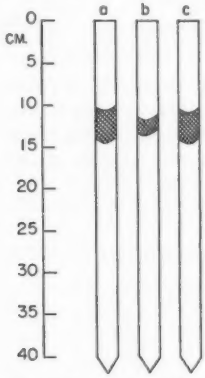

FIG. 3. Paper chromatograms of Strip a, authentic methyl-[(testosterone-17β)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate (50γ); Strip b, acetylated and esterified product from liver-testosterone incubations; Strip c, mixed chromatogram of Strips a (50 γ) and b (50 γ).

tity. The compounds were chromatographed in a ligroin-ethylene glycol system. The chromatogram was stained with Zimmermann's reagent, giving a deep blue color.

DISCUSSION

The evidence presented here shows that the glucuronic acid conjugate of testosterone formed during the incubation *in vitro* with rat liver after acetylation and esterification is identical with synthetic methyl-[(testosterone-17β)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid] uronate.

The chemical characterization of a hormone and its metabolites is a requirement for any accurate understanding of the metabolism of the hormone. In the case of testosterone, the conjugates of its metabolites were known long before testosterone glucosiduronic acid was discovered. It is therefore interesting to consider that the first step in the metabolism of

testosterone, the primary testicular androgen, is its conjugation at position 17 with glucuronic acid. The possible significance of this conjugation has been discussed elsewhere and its organ site is not limited to the liver (9).

SUMMARY

Testosterone glucosiduronic acid formed during the incubation of testosterone with rat liver was converted to its polyacetyl methyl ester. The ultraviolet and infrared absorption spectra of this substance matched the corresponding spectra of pure authentic methyl-[(testosterone-17β)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate. This finding provides finite proof of the structure of testosterone glucosiduronic acid as testosterone-17β-D-glucopyranuronoside.

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COMPARATIVE ABILITY OF SOME STEROIDS AND THEIR ESTERS TO ENHANCE THE RENAL β-GLUCURONIDASE ACTIVITY OF MICE*

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(Received for publication, December 10, 1957)

In earlier studies from this laboratory (1–3), it was demonstrated that on parenteral administration certain steroids, particularly those closely related to testosterone, possessed the ability to enhance the kidney β-glucuronidase activity of various strains of mice. Fishman, Artenstein, and Green (3) established experimental conditions which made it possible to obtain uniform and reproducible figures for mouse kidney β-glucuronidase activity in the Ajax strain of mice. A large number of steroids were screened. Androgens as a group were found to increase renal β-glucuronidase, seven out of forty-nine pregnane and corticoid derivatives showed weak activity, and estrogens and progesterone were without effect. With use of testosterone as the reference steroid (which gave a 3-fold response), they found that the following changes in the molecule enhanced the potency: (1) esterification of the 17β-OH, (2) introduction of a 17α-CH_3 group, (3) reduction of the Δ^4 double bond, and (4) elimination of the 19-CH_3 group. The following alterations reduced potency: replacement of the 17β-OH (1) by a 17α-OH, (2) by a 17-ketone, and (3) by introduction of an 11α-, 14α-, or 6β-OH group.

In the Δ^5-androstenediol series, enhancement of activity was seen in the 17α-CH_3 compound, the potency of which was reduced by blocking the 17β-OH group or by oxidizing it to a 17-keto group.

In the present report, conditions are described which make the assay more reliable and significant for steroids highly potent with regard to the renal β-glucuronidase response. By this means, it was discovered that esters of testosterone had a much higher order of activity than that shown by unesterified testosterone. Moreover, the study of newer compounds by the "screening" method has yielded findings which bear on the relationship between structure and activity of some protein-anabolic steroids.

* Aided by grants-in-aid Nos. P-106 and P-107, American Cancer Society, Inc., New York.

† Summer Scholar, Aid for Cancer Research, 1956; Polio Summer Scholar, 1957.

Materials

Animals—Ajax male mice were obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine, and were placed on experiment at a body weight of 23 ± 3 gm.

Diet—The animals were maintained on a synthetic diet (25 per cent protein, 25 per cent fat) described earlier (3).

Steroids—The 16-oxygenated steroids were kindly supplied by the Nepera Chemical Company, the 17-alkylated nortestosterone compounds by

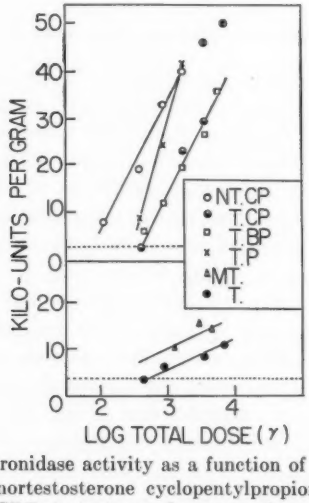

FIG. 1. Renal β-glucuronidase activity as a function of log total dose of steroid administered. NTCP, nortestosterone cyclopentylpropionate; TCP, testosterone cyclopentylpropionate; TBP, testosterone bromopropionate; TP, testosterone propionate; MT, methyltestosterone; and T, testosterone.

Searle and Company, and the remainder of the steroids were generously donated by The Upjohn Company. Solutions of each were prepared in peanut oil (10 mg. per ml.) and the conditions for steroid testing were as described earlier (3).

EXPERIMENTAL

Investigation of High Potency Steroids—Log-dose relationships have been established for certain high potency steroids (Fig. 1). It may be seen that nortestosterone cyclopentylpropionate (NTCP) was as active as testosterone propionate at high dosage, whereas at lower doses of these steroids, NTCP is the more potent compound. Testosterone cyclopentylpropionate (TCP) and testosterone bromopropionate (TBP) showed identical po-

tencies at both low and high dosages. These are of a different magnitude from that of the first pair of steroids mentioned.

Methyltestosterone (MT) and testosterone (T) exhibit linear log-dose relationships, with MT being more active than T.

Relation of Response to Chemical Structure of Steroids—Table I lists the kidney β-glucuronidase factors obtained for twenty steroids and provides the statistical evaluation of these data with regard to findings considered significant in this paper.

In the 16-oxygenated steroid series, none of the androstane and Δ^4- and Δ^5-androstene derivatives having either a 16-hydroxyl or a 16-ketone group showed any significant activity. On the other hand, the presence of a 16β-OH or a 16-keto group on the estrane molecule did enhance the renal β-glucuronidase response, whereas, when the 16-OH was in the α position, there was no increased activity (Experiments 1, 2, and 3).

The testosterone derivatives tested were chiefly related to 19-nortestosterone and 17-methyltestosterone. Thus, the addition of either a 17-ethyl or 17-methyl group to nortestosterone increased the kidney β-glucuronidase factors to 7.8 and 7.2, respectively, from the level of 4.0 (nortestosterone). The enhancement resulting from the introduction of a 17-methyl group was observed previously in the pair of steroids, testosterone *versus* 17-methyltestosterone. It is now noted that the introduction of an 11β-OH group decreased the activity of methyltestosterone markedly. The further addition of a fluorine atom at the 9α position forming 9αF-11β-OH-17-methyltestosterone counteracts the inhibiting effect of the 11β-OH group. It is interesting that the analogous 11-keto compound gave only a moderate response. The replacement of the 17β-OH by a 17β-CH_3 group in Δ^5-androstene-3β,17β-diol caused no change in activity.

DISCUSSION

Under physiological circumstances it would appear that mouse renal β-glucuronidase is under the control of a number of factors, including especially testicular androgens, the secretion of which is governed by interstitial cell-stimulating hormone (4). For mice of a pure inbred strain, the kidney enzyme levels are unusually constant and reproducible, a fact which may be determined genetically (5). Moreover, the ability of exogenous steroids to elevate the renal β-glucuronidase titer above its normal level is a circumstance which has favored the development of a biochemical assay for androgens.

This response is considered to be reasonably specific for androgens inasmuch as estrogens, progesterone, and a large number of pregnane derivatives gave negative results. In the present report the new facts which bear on the nature of the renal β-glucuronidase response are now discussed.

TABLE I

Relation of Response to Chemical Structures of Steroids

Steroid category	Experiment No.	Steroid	Kidney β-glucuronidase factor*	Statistical significance of differences			
				Experiment No.	vs. Experiment No.	t	P
Estrogens	1	Estradiol-3,17β	0.8				
	2	Estradiol-3,16β	2.8, 2.8				
	3	Estradiol-3,16α	1.0	2	3	3.5	<0.01
	4	16-Ketoestrone	1.9	2	4	3.75	<0.01
Testosterone and derivatives	5	19-Nortestosterone	4.1				
	6	17-CH_3-Nortestosterone	7.2	5	6	13.1	<0.01
	7	17-C_2H_5-Nortestosterone	7.8	5	7	12.5	<0.01
	8	17-CH_3-Testosterone	6.3				
	9	11β-OH-17-CH_3-Testosterone	1.9	8	9	7.8	<0.01
	10	9αF-11β-OH-17-CH_3-Testosterone	5.3	10	11	5.0	<0.01
	11	9αF-11-Keto-17-CH_3-testosterone	2.3	8	11	7.2	<0.01
	12	17β-CH_3-Δ^5-Androsten-3β-ol	2.1				
16-Oxygenated androdrogens	13	Δ^5-Androstene-3β,16β-diol	1.3				
	14	Androstan-3α-ol-16-one	1.6				
	15	Androstane-3α,16β-diol	1.3				
	16	Δ^5-Androstene-3β,16β-diol	1.0				
	17	Δ^5-Androsten-3β-ol-16-one	1.2				
	18	Δ^4-Androstene-3,16-dione	1.1				
	19	3β-Methoxy-Δ^5-androsten-16-one	1.1				
17-Oxygenated androgens	20	No steroid	1.0				
	21	Testosterone	2.9				
	22	17-CH_3-Testosterone	6.3	21	22	3.70	<0.01
	23	17β-CH_3-Δ^5-Androsten-3β-ol	2.1	20	23	8.50	<0.01
	24	Δ^5-Androstene-3β,17β-diol	2.0				
	25	17α-CH_3-Δ^5-Androstene-3β,17β-diol	9.5	24	25	6.5	<0.01

* Defined as the mean kidney β-glucuronidase activity of the test group divided by 3100, which is the value established for control groups not receiving steroid (Fishman *et al.* (3)).

Biochemical Assay of Steroids, Highly Active in Eliciting Renal β-Glucuronidase Response—Our experience with a number of testosterone esters indicated that they all gave kidney glucuronidase factors in the range of 10 to 17, never greater than 17. At that time, it was suspected that the response took place at the high dosage plateau of the dosage-response curve which was only slightly increased by a further increase in dosage. In testing highly active compounds, it was found that, the higher the potency, the greater the differences in the mean between that steroid and another must be if the two are to be considered unequal because there is 10 to 20 per cent variation in the data for each steroid. The assay of such steroid esters at lower dosage as was given here did make for greater reliability.

TABLE II

Potency of Testosterone Esters

	Total Dosage,* D	Kidney β-glucuronidase factor (3)	Response units
	mg.		*1/D × 24*
Control (no steroid)	0	1.0†	
Testosterone	8	3.0	3.0
Methyltestosterone	1	6.3	24.0
Testosterone propionate	0.4	16.3	60.0
" cyclopentylpropionate	0.8	12.4	30.0
" bromopropionate	0.8	11.4	30.0
Nortestosterone cyclopentylpropionate	0.16	15.7	150.0

The figure in parentheses is a bibliographic reference.

* Required to produce a kidney assaying 10,000 units per gm., according to Fig. 1.

† Mean kidney β-glucuronidase activity = 3100 ± 300 units per gm.

The free steroids such as testosterone and methyltestosterone gave linear dosage-response curves which were different in slope from the testosterone esters.

In order to make comparisons of potency in even an elementary fashion, a definition of a tentative renal β-glucuronidase response unit is required. Accordingly, the reciprocal of the amount in mg. of steroid administered, under the standard conditions described, required to produce a kidney assaying 10,000 units per gm., multiplied by 24, represents the potency of the steroid in eliciting the renal β-glucuronidase response. The factor of 24 was chosen in order to adjust the units to testosterone as the reference compound where the kidney β-glucuronidase factor for testosterone is taken as 3. This has been done for the compounds in Fig. 1 and the comparative data appear in Table II.

It is clear that the new way of expressing potency increases the range of

TABLE III

Comparison of Biological Responses to Testosterone Propionate and Testosterone (Kochakian (6))

Investigator	Animal material	End point of response	TP	T	Ratio, $\frac{TP}{T}$
Meyer	Parabiotic rats	Pituitary inhibition	81.3 %	59 %	1.38
Hertz	" "	Dosage for complete pituitary inhibition	15 mg.	75 mg.	5.0
Dorfman	Chicks	Increase in combined weight (40 mg. dose;	139 %	172 %	0.81
		160 mg. dose)	158 %	362 %	0.44
Leathem	Immature rats	Increase in seminal vesicle weight 72 hrs. after single injection	20 % (0.25 mg.)	70 % (3.4 mg.)	Estimated 44.0
Kochakian	Castrated mice	30 days increase in body weight (% of original body weight)	253 %	229 %	1.1
Gordon	Castrated rats	% of control weight of levator ani muscle	280 % (1 mg. per day)	190 % (0.1 mg. per day)	0.15
Meyer	Immature rat	7 day increase in weight of ventral prostate	810 %	380 %	2.13
	" "	21 day increase (ventral prostate)	114 %	98 %	1.16
	Adult rat	" "	130 %	114 %	1.14
	Immature rat	21 day increase in weight of seminal vesicle	235	171	1.37
	Adult rat	" "	107	100.01	1.07
	Immature rat	7 day increase in weight of levator ani	209 %	142 % (0.35 mg.)	1.47
	" "	21 day increase in weight of levator ani	118	106 (1.4 mg.)	1.11
	Adult rat	" "	105	112	0.94
	Immature rat	21 day % inhibition of testis	82.0	52.5 (1.4 mg.)	1.56
	Adult rat	21 day % inhibition of testis	13.0	5 (1.4 mg.)	2.6
This paper	Ajax mice	Production of kidney β-glucuronidase 10,000 units per gm.	60	3	20.0

response to such an extent that high potency testosterone esters can be graded on the same scale as less potent ones. Moreover, the relationship of the potency of one steroid to another has changed; *e.g.*, the ratio of kidney β-glucuronidase factor for methyltestosterone *versus* testosterone is approximately 2.0, whereas the ratio of response units for the two steroids is 8. Also, nortestosterone cyclopentylpropionate, which was not expected to be more potent than testosterone propionate on the basis of the "screening" data, proved to be otherwise, potency 150 *versus* 60.

In this connection it will be recalled that the previous kidney β-glucuronidase factor (3) served as a means of grading steroids into three categories of activity. These were kidney β-glucuronidase factors of 2 to 4, 5 to 10, and 10 to 16. The present measure of kidney β-glucuronidase response provides a more quantitative assay of steroids and is recommended for the further study of compounds discovered to be active after a "screening" procedure.

A review of comparisons of testosterone propionate *versus* testosterone by other assay methods (Table III) shows that, for many of these, testosterone propionate did not markedly enhance the response above that observed for testosterone. This is in agreement with the generally held opinion that the outstanding characteristic of the esters of testosterone is that they prolong the duration of the biological effect of testosterone but do not enhance it. This contrasts with the 20-fold difference noted in the renal β-glucuronidase response for this pair of steroids.

Relation of Structure to Activity—From our previous work it appeared that the functional groups in ring D of the steroid molecule were most important in determining the activity of the compound. Particularly interesting correlations were found for position 17. The study of 16-oxygenated steroids now shows unexpected activity in 16-ketoestrone and in estradiol-3,16β. These substances are reputed to be weak estrogens. When the configuration at position 16 was α instead of β, the compound was inactive. This is analogous to the results with 17-epitestosterone and testosterone (3).

It was reported earlier that the introduction of a 17-methyl group enhanced activity, *e.g.* methyltestosterone *versus* testosterone. Additional examples of enhancement of activity by 17-alkylation are reported in this paper (Table I). Moreover, this result can be influenced by the nature of substituents at positions 9 and 11. Thus, the introduction of an 11β-OH group into methyltestosterone reduces the activity to one-third of its original activity. Also, the presence of a halogen atom (fluorine) at position 9 was a factor which had an influence on the activity of the steroids.

Renal β-glucuronidase response to androgens (3) was not related particularly to the virilizing potency of the steroid. Rather, it seemed to be a

function of compounds reputed to possess a higher ratio of protein-anabolic to virilizing properties. Among the present series of compounds which show good potencies are the newer protein-anabolic agents (19-nortestosterone, 17α-methyl nortestosterone). Accordingly, our previous impression appears to be further supported by the present data.

SUMMARY

It has been possible to arrive at a more reliable estimate of the potency of esters of testosterone by means of the mouse renal β-glucuronidase response. This was accomplished by means of a dose-response curve in which greatly reduced amounts of steroid were employed. The potency of a steroid in eliciting the β-glucuronidase response was defined as 24 times the reciprocal of the dose required to produce a kidney assaying 10,000 units per gm. The standard of reference is testosterone. According to this measure, testosterone propionate exhibits a potency of 60 and testosterone one of 3.0. Nortestosterone cyclopentylpropionate was the most potent compound in eliciting the response (potency = 150). The existence of as marked a difference in response to testosterone propionate and its other esters *versus* testosterone is reported here for the first time.

3,16β-Estradiol and 16-ketoestrone gave 2- to 3-fold increases in renal β-glucuronidase.

The introduction into nortestosterone of a 17-methyl or 17-ethyl group was found to enhance its potency as measured by the renal β-glucuronidase response.

We wish to express our gratitude to a number of medical students who have assisted at one time or another in this investigation, particularly Mr. Edward Hogan, William Monafo, and John Laverty.

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PURIFICATION AND PROPERTIES OF D-XYLULOKINASE IN LIVER

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(Received for publication, January 8, 1958)

In 1914, Levene and La Forge (1) identified L-xylulose as the sugar excreted in the urine of certain individuals with a benign metabolic lesion known as "essential pentosuria." No further information on the origin of this pentose was forthcoming until 1935, when Enklewitz and Lasker (2) reported the highly significant observation that the ingestion of D-glucuronolactone by these patients resulted in a large increase in the urinary pentose output. Shortly thereafter, Larson *et al.* (3) demonstrated that L-xylulose could serve as a precursor for glucose in diabetic dogs, although, with the techniques available at that time, they were unable to show significant utilization in normal animals. That this compound might function as a normal metabolic intermediate was first demonstrated by the work of Touster *et al.* (4), who reported that D-glucuronolactone was converted to L-xylulose by non-pentosuric human and guinea pigs.

At this point, a new approach to the problem was made possible by the combined findings of a group of independent investigators. In 1954, D-xylulose 5-phosphate[1] was identified as an enzymatic product of ribose 5-phosphate metabolism in spleen by Ashwell and Hickman (5). The significance of this compound was quickly established by Srere *et al.* (6), Horecker *et al.* (7), and their coworkers. They found that D-xylulose 5-phosphate was the true substrate for the transketolase-catalyzed formation of sedoheptulose 7-phosphate and thus an intermediate in the conversion of ribose 5-phosphate to glucose 6-phosphate by means of the pentose cycle. Further studies by Touster *et al.* (8) and by Hollmann and Touster (9) revealed the presence of an enzyme system in guinea pig liver mitochondria capable of reversibly reducing both L- and D-xylulose to a common intermediate, xylitol, thereby providing a mechanism for the interconversion of the stereoisomers of this ketopentose. From the above findings, Hollmann and Touster (10) proposed the following sequence

* Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry at Georgetown University, Washington, D. C.

[1] The following abbreviations are used: Xu5P, xylulose 5-phosphate; G6P, glucose 6-phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; TEA, triethanolamine; MEA, monoethanolamine; DPN, diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

for the conversion of L-xylulose to glucose:

$$\text{L-Xylulose} \xrightleftharpoons{\text{TPNH}} \text{xylitol} \xrightleftharpoons{\text{DPN}} \text{D-xylulose} \xrightarrow{\text{ATP}}$$

$$\text{D-Xu5P} \xrightleftharpoons[\text{cycle}]{\text{pentose}} \text{G6P} \rightleftharpoons \text{glucose}$$

The presence of an enzyme system in mammalian tissue that is capable of phosphorylating D-xylulose, the only missing step in the above scheme, was reported in a brief communication from this laboratory (11). This finding indicated that mammalian tissue does possess the complete enzymatic structure necessary to carry out the conversion of L-xylulose to glucose as postulated by Touster. The present paper describes further experiments to support the validity of the above reaction scheme and provides a more detailed study of the purification and properties of the enzyme D-xylulokinase.

Materials and Methods

Materials—D-Xylulose was prepared by the action of *Acetobacter suboxydans* on D-arabitol (12). We are indebted to Dr. H. B. Wood and Dr. L. Stewart of this Institute for generously providing the synthetic pentitol and for aid in growing the organism. L-Xylulose and D-ribulose were obtained by the pyridine isomerization of L-xylose and D-arabinose, respectively (13, 14). In both cases, the crude reaction product was treated with bromine in order to convert the unchanged aldopentose to the corresponding aldonic acid, which was then removed with Duolite A-4. The effluent was concentrated *in vacuo* at 40° to a brown syrup and stored in an evacuated desiccator at 0°. Pure sedoheptulose was regenerated from the crystalline hexaacetate, provided by Dr. N. Richtmyer, by treatment with sodium methoxide. Phosphoenol pyruvate was a gift from W. E. Pricer, Jr., of this Institute. Subsequent batches were synthesized from β-chlorolactic acid (15). Creatine phosphate and creatine phosphate transferase were prepared by Dr. Simon Black of this Institute. Alumina Cγ was prepared as described by Willstätter *et al.* (16), and the calcium phosphate gel by Keilin and Hartree (17). Both gels were allowed to age from 6 months to 1 year before use.

Potato phosphatase was purified by the method of Kornberg.[2] The twice recrystallized lactic acid dehydrogenase, which contained phosphoenolpyruvic kinase, was supplied by the Worthington Biochemical Corporation. All other substrates and cofactors were obtained from commercial sources.

Analytical Procedures—Xylulokinase activity in the crude extract was

[2] Prepared from an unpublished procedure of Dr. A. Kornberg.

determined by measurement of the D-xylulose remaining in the supernatant solution after precipitation of the phosphorylated reaction products with barium and ethanol. The assay was proportional to increasing amounts of enzyme (Fig. 1).

In more purified fractions, it was found convenient to follow the xylulokinase activity by measurement of the ADP formed in the kinase reaction by the method of Kornberg and Pricer (18). Excellent correlation was

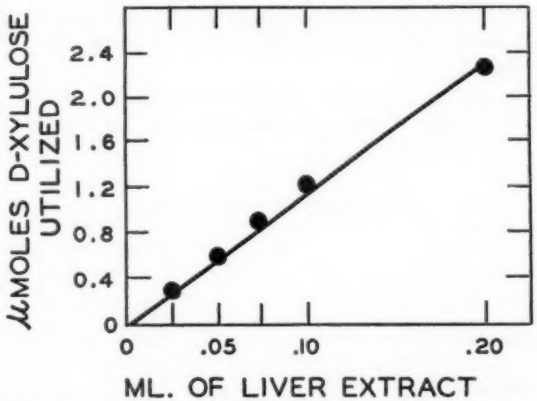


FIG. 1. Assay method for D-xylulokinase. The reaction mixture contained 40 μmoles of TEA buffer, pH 7.4, 5 μmoles of $MgCl_2$, 5 μmoles of ATP, 3.0 μmoles of D-xylulose, and 2.0 μmoles of cysteine in a total volume of 0.5 ml. Enzyme additions were as indicated. The mixture was incubated at 37° for 10 minutes and then stopped by addition of 0.2 ml. of 1 M barium acetate and 3 ml. of ethanol. The supernatant solution was assayed directly for disappearance of xylulose. A unit is defined as that amount of enzyme capable of phosphorylating 1 μmole of D-xylulose per 10 minutes under the above conditions.

obtained with both methods. Colorimetric analyses of mixtures of the free and phosphorylated pentoses have been described previously (19).

Phosphorus analyses were performed according to the King modification of the Fiske-Subbarow procedure (20), and protein analyses by the optical method of Warburg and Christian (21) and by the phenol method (22).

Enzyme Purification

The calf liver acetone powder was prepared and stored as described previously for spleen (19). Upon standing, the dried enzyme powder loses activity and the purification procedure becomes difficult to repeat. For the best results, the acetone powder should be less than 2 weeks old.

Water Extract—10 gm. of calf liver acetone powder were stirred with

200 ml. of distilled water at room temperature for 20 minutes, and the insoluble material was removed by centrifugation. The crude enzyme extract is quite unstable and will not withstand freezing or prolonged storage at 0° ("Water extract," 170 ml.).

Ammonium Phosphate—To 170 ml. of the above extract were added 44.2 gm. of dibasic ammonium phosphate, and the solution was allowed to stand for 30 minutes at 0°. The precipitate was collected by centrifugation in the cold and dissolved in 30 ml. of distilled water, the enzyme was dialyzed overnight at 0° against 4 liters of 0.01 M K_2HPO_4, and a small amount of insoluble material was removed by centrifugation ("Ammonium phosphate," 44 ml.).

Gel Step I—To the dialyzed enzyme containing 1.56 gm. of protein were added 1.56 gm. of calcium phosphate gel (dry weight). In this, as in the following gel steps, the purification obtained was by adsorption of inactive material, and the gel suspension was centrifuged to minimize volume increases. Thus, 260 ml. of a gel preparation containing 6.0 mg. of calcium phosphate per ml. were centrifuged, and the gel was suspended directly in the enzyme solution. After standing for 15 to 20 minutes in the cold, the solution was centrifuged and the gel rejected. The supernatant solution, containing a total of 510 mg. of protein, was then treated with another 510 mg. of calcium phosphate gel, as described above. At this point, further addition of calcium phosphate proved ineffective. However, considerable purification could be obtained by a third treatment with alumina Cγ gel. Here, as above, the best results were obtained when the gel to protein ratio was kept at 1:1 ("Gel step I," 46 ml.).

TABLE I

Purification of D-Xylulokinase

	Total units	Specific activity
		units per mg. protein
Water extract	5450	0.85
Ammonium phosphate	3390	2.2
Gel step I	1590	9.0
Ammonium sulfate	1480	12.5
Gel step II	780	41.0

Ammonium Sulfate—To the colorless gel supernatant solution was added an equal volume (46 ml.) of cold saturated ammonium sulfate, adjusted to pH 8.0, with concentrated ammonium hydroxide. After standing for several hours in the cold, the precipitate was collected and dissolved in a small amount of cold distilled water. At this stage of puri-

fication, the enzyme was stable at −12° for over a month ("Ammonium sulfate," 6.0 ml.).

Gel Step II—To 6.0 ml. of the ammonium sulfate fraction containing 19.5 mg. of protein per ml. were added 117 mg. of alumina Cγ gel. After standing for 15 to 20 minutes in the cold, the suspension was centrifuged. This supernatant solution, containing 6.2 mg. of protein per ml., was again treated with an equal weight of alumina Cγ gel, and the gel was rejected as above. The final preparation was about 50-fold purified. The yield and specific activity of each step are given in Table I ("Gel step II," 6.0 ml.).

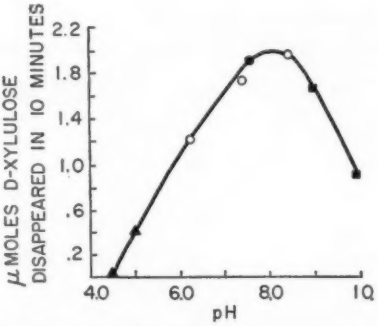


FIG. 2. pH optimum of D-xylulokinase. Assay conditions were as described in Fig. 1. ▲, acetate; ○, phosphate; ●, TEA; ■, MEA.

Properties of D-Xylulokinase

Stability—Preparations of D-xylulokinase are generally unstable and lose most of their activity within a few days after preparation. It is therefore advisable to proceed to the ammonium sulfate step without interruption. At this point, the enzyme may be stored at −12° for over a month. However, repeated freezing and thawing are to be avoided. The enzyme can best be stored in small aliquots which can then be carried through the final gel step separately. The addition of sulfhydryl compounds was ineffective in the stabilization of the enzyme. Attempts to preserve the preparation as a lyophilized powder met with variable success.

pH Optimum—The phosphorylation of D-xylulose proceeds maximally in the pH range of 7.5 to 8.5, as shown in Fig. 2. Routine assays were carried out at the lower pH in order to minimize the alkaline-catalyzed destruction of the reaction product.

Specificity—The enzyme appears to be specific for D-xylulose. Among

the substrates tested and found inactive were L-xylulose, D- and L- ribulose, D- and L-xylose, D-ribose, D-fructose, and D-altroheptulose (sedoheptulose).

Inhibitors and Activators—The kinase appears to be a sulfhydryl enzyme in that its activity was completely blocked by 10^{-4} M *p*-chloromercuribenzoate, but was fully restored by the addition of 10^{-3} M cysteine. Fluoride and iodoacetate at comparable concentrations were without effect. A stimulation due to magnesium was evident, even in the crude extract, whereas more highly purified preparations were completely inactive in the absence of this metal. Cobalt, manganese, calcium, iron, and zinc were unable to replace magnesium.

TABLE II*

Effect of ATP:$MgCl_2$ Ratio on D-Xylulokinase

ATP	ADP	$MgCl_2$	Creatine phosphate and transferase	D-Xu5P formed
5	—	5	—	1.40
5	—	20	—	1.20
20	—	5	—	0.60
20	—	20	—	1.40
5	0.9	5	—	1.23
5	2.5	5	—	0.77
5	5.0	5	—	0.74
5	5.0	20	—	0.78
5	—	5	+	2.10

* Assay conditions were as described in Fig. 1. All figures are given in micromoles.

Optimal activity was obtained when a $MgCl_2$-ATP ratio of at least 1 was maintained. It can be seen from the data in Table II that the marked inhibition caused by a 4-fold increase in ATP was completely overcome by an equal amount of $MgCl_2$. Addition of ADP caused a similar inhibition, which, however, was unaffected by increasing concentrations of $MgCl_2$. When the accumulation of ADP was prevented by the inclusion of creatine phosphate and its transferase in the reaction mixture, an appreciable stimulation of activity was noted.

Effect of Substrate Concentration—In order to investigate the effect of substrate concentration on the kinase reaction, it was necessary to employ an assay system more sensitive than that used in the major portion of this work. The method used was developed by Dr. J. Hurwitz of Washington University and involved the measurement of the amount of Xu5P formed in the incubation mixture. Phosphoketolase was allowed to react in the presence of inorganic P^{32} with Xu5P to give P^{32}-labeled acetyl phosphate. The acetyl P^{32} was then determined quantitatively by adding ADP

and acetokinase, by adsorbing the ADP-P^{32} formed on charcoal, and measuring the activity. In this manner it was possible to determine extremely small amounts of Xu5P. The over-all reaction is formulated as follows:

$$\text{Xu5P} + \text{inorganic } P^{32} \xrightarrow{\text{phosphoketolase}} \text{acetyl } P^{32} + \text{triose phosphate}$$

$$\text{Acetyl } P^{32} + \text{ADP} \xrightarrow{\text{acetokinase}} \text{acetate} + \text{ADP-}P^{32}$$

The incubation mixture, which contained 5.0 μmoles of ATP, 5.0 μmoles

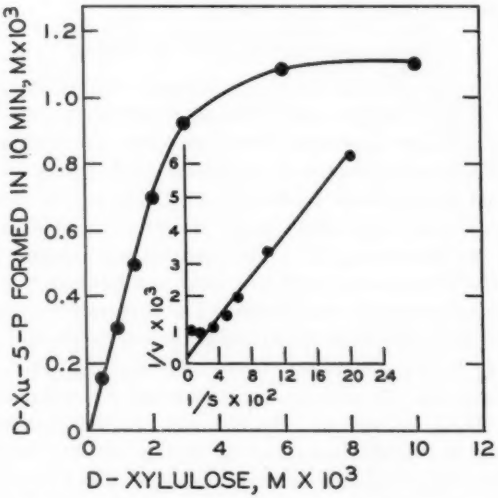


FIG. 3. The effect of D-xylulose concentration on the reaction velocity. The assay conditions and methods are described in the text.

of $MgCl_2$, 40 μmoles of TEA buffer at pH 7.0, 1.0 μmole of cysteine, and 0.075 ml. of the crude extract in a total volume of 0.5 ml., was incubated for 10 minutes at 37°, boiled for 1 minute to stop the reaction, and then chilled. D-Xylulose concentrations were as indicated in Fig. 3. Xu5P concentration was determined by the phosphoketolase-acetokinase reaction.

0.01 and 0.22 ml. aliquots of the incubation mixture, 0.76 μmole of P_i (1.94×10^{-4} c.p.m.), 25 μmoles of succinate buffer at pH 6.0, 10 μmoles of $MgCl_2$, 0.025 μmole of thiamine pyrophosphate, 0.9 μmole of ADP, 0.005 ml. of 0.03 M glutathione containing 2.5×10^{-3} M Versene, 60 units of phosphoketolase, and 1.2 units of acetokinase, in a total volume of 1.3 ml., were incubated for 10 minutes at 37°. The reaction was stopped

by the addition of 1.0 ml. of 2.5 per cent cold perchloric acid. To this solution were added 20 mg. of acid-washed charcoal (Darco) to adsorb the ADP-P^{32}. After standing 5 to 10 minutes at 0°, the sample was diluted to 10 ml. with the perchlorate solution and centrifuged. This process was repeated two more times and the charcoal transferred to a sintered glass planchet and counted. The results are shown graphically in Fig. 3. An attempt was made to determine the affinity constant for D-xylulose by means of a Lineweaver-Burk plot of the data, and a K_m of 4×10^{-2} M was obtained. However, because of appreciable substrate inhibition, this figure must be regarded as an approximation.

Preparation and Isolation of Product

When the enzyme preparation was incubated with D-xylulose and ATP, a mixture of ribose, ribulose, and xylulose 5-phosphate resulted, thereby indicating the presence of phosphoribose isomerase and phosphoketopentoepimerase. The reaction mixture, containing 1000 μmoles each of D-xylulose, ATP, and $MgCl_2$, was incubated with 100 mg. of enzyme ("Ammonium sulfate" step) in a total volume of 150 ml. of 0.02 M TEA buffer at pH 7.6 for 30 minutes at 37°. The reaction was stopped by the addition of 7.5 ml. of 60 per cent perchloric acid, the mixture was centrifuged, and the precipitate washed with water. The supernatant fluid and washings were combined, adjusted to pH 2.2 with 5 N KOH, and treated with 100 ml. of a 30 per cent charcoal (Darco) suspension. The charcoal was allowed to stand for 30 minutes, was removed by filtration, and then washed with 60 ml. of water. The efficiency of the charcoal treatment was checked by the determination of the optical density at 260 mμ after filtration. The combined filtrate and washings (400 ml.) were adjusted to pH 6.8 with 1 N KOH, and the sugar phosphates were precipitated by the addition of 5 ml. of 1 M barium acetate and 4 volumes of ethanol. The precipitate was collected, washed with ethanol and ether, and dried. The dry barium salt, weighing 206 mg., contained 360 μmoles of total phosphate and 30 μmoles of inorganic phosphate. The results of the analysis of the reaction product are shown in Table III.

Further confirmation of the identity of the sugar phosphates was obtained by enzymatic dephosphorylation and isolation of the sugars as their borate complexes. The barium salt was dissolved in dilute H_2SO_4, and the barium sulfate removed by centrifugation. To the supernatant fluid, adjusted to pH 5.0, were added 20 mg. of $MgCl_2$ and 68 mg. of sodium acetate. The reaction flask was placed in a water bath at 37°, and 15 ml. of potato phosphatase were added in 5 ml. portions over a 3 hour period. At the end of this time all of the organic phosphate had been

hydrolyzed. The sugar solution was deionized on a mixed bed resin of Duolite A-4 and Amberlite IR-120.

The free pentoses were made 0.01 M with potassium borate. They were added to a Dowex 1 borate column (2.2 × 30 cm.), and washed with 50 ml. of 0.01 M borate. The rate of flow was adjusted to about 1 ml. per minute, and 12 ml. fractions were collected. Xylulose (tubes 36 to 65) and ribose (tubes 66 to 100) were eluted from the column with 0.02 M borate. The borate concentration was then increased to 0.05 M, and

TABLE III

Assay of D-Xylulokinase Reaction Products

Component	Phosphate ester formed	Dephosphorylated sugar recovered
	μmoles	*μmoles*
Xylulose	150	100
Ribulose	100	67
Ribose	80	55
Total	330	222

TABLE IV

Optical Rotation of Dephosphorylated Reaction Product

Fraction	$[\alpha]_D^{24}$ observed	$[\alpha]_D^{24}$ authentic sugar
	degrees	*degrees*
Xylulose	−33.2	−33.1
Ribose	−22	−23.2
Ribulose	−15	−16

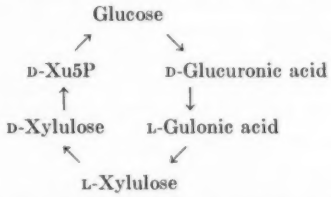
the ribulose fraction was collected (tubes 151 to 175). The tubes in each peak were pooled separately and treated batchwise with Amberlite IR-120 (H^+) in order to convert the potassium salt to free boric acid. The resin was removed by filtration, and the filtrate was concentrated to a syrup *in vacuo* at 40°. The syrup was dissolved in 75 ml. of methyl alcohol, and the methyl borate was removed by distillation *in vacuo* (23). This process was repeated three times. The syrups were dissolved in water, treated with charcoal, and filtered. Water-clear solutions of the sugars were obtained. Recovery of the dephosphorylated pentoses is shown in Table III.

Xylulose was identified by its characteristic reaction in the cysteine-carbazole and orcinol reaction (19). Upon paper chromatography with

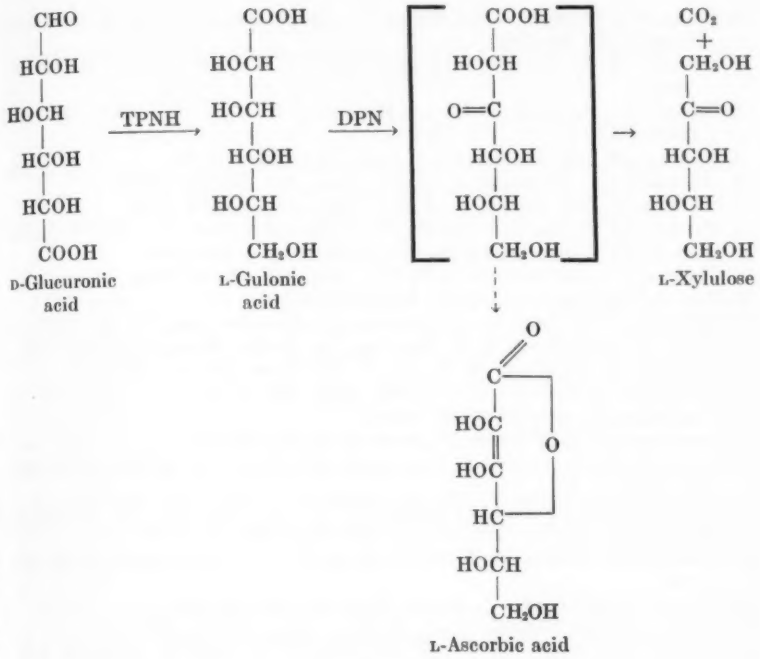
phenol-water (9:1) and amyl acetate-acetic acid-water (3:3:1), the xylulose fraction gave a single spot corresponding to known xylulose. The optical rotation of the recovered sugar is shown in Table IV. The xylulose fraction formed a crystalline *p*-bromophenylhydrazone that melted at 126–128° and remained unchanged when mixed with a similar derivative prepared from authentic xylulose. The identity of the ribose and ribulose fractions was checked by optical rotation, paper chromatography, and colorimetric analysis (Table IV).

DISCUSSION

The data presented here for the metabolism of D-xylulose by means of a specific D-xylulokinase complete the sequence of known enzymatic reactions in animal tissues whereby L-xylulose is converted to glucose. This is in accord with and strongly supports the original postulation of Hollmann and Touster that D-xylulose, resulting from the reduction of L-xylulose to xylitol and the subsequent oxidation of the xylitol, enters the pentose cycle as D-Xu5P. An important consequence of these observations lies in the tentative establishment of a new cyclic pathway for the oxidation of glucose. Thus, glucuronic acid, which is known to be derived directly from glucose without prior cleavage of the carbon chain (24), has recently been shown to be the precursor of L-xylulose in mammalian tissues (25–28). The cyclic nature of these reactions may be indicated briefly as follows:



Significantly, in addition to giving rise to L-xylulose, both D-glucuronic and L-gulonic acids have been identified as precursors of ascorbic acid in various animals other than man and the guinea pig (29, 30). A more detailed inspection of the reaction sequence has prompted the suggestion of 3-keto-L-gulonic acid (3-keto-L-galactonic acid) as the hypothetical intermediate in the formation of L-xylulose from L-gulonic acid (25, 31). From structural considerations, it can be seen that enolization followed by lactonization could readily lead to the formation of ascorbic acid, and that decarboxylation would yield L-xylulose. Consequently, the significance of this compound lies in the fact that it may well represent a point of bifurcation of two diverse metabolic pathways, one of which, ascorbic acid formation, is missing in man, monkey, and guinea pig.



SUMMARY

Evidence is presented for the occurrence in mammalian liver of a specific D-xylulose-phosphorylating enzyme that catalyzes the reaction:

D-Xylulose + ATP → D-Xu5P + ADP

This enzyme has been isolated in a partially purified form, and a study of its properties was undertaken. The data support and document further the hypothesis of Hollmann and Touster that L-xylulose is converted to glucose by a series of reactions involving the pentose cycle. The significance of this finding in relation to a new cyclic pathway for glucose metabolism is discussed.

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IMMEDIATE EFFECTS OF INSULIN ON GLUCOSE UTILIZATION IN NORMAL RATS *

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(Received for publication, December 27, 1957)

At present a wealth of data may be cited in support of the concept that insulin promotes the utilization of glucose, either by increasing cellular permeability or by increasing the speed of a rate-limiting intracellular reaction (1–6). However, none of these numerous studies has clearly established an enhanced glucose utilization as the cause of the *immediate* hypoglycemic action of this hormone. This uncertainty has been emphasized recently as a result of isotopic tracer studies, carried out in our own and other laboratories, showing that *part* of the immediate hypoglycemic action of insulin is due to inhibition of entry of glucose into the blood stream, presumably by suppression of hepatic glucose output (7–10).

It was recognized by the earliest investigators of insulin action (11) that the most direct and least ambiguous test of the immediate effects of this hormone would be to find out what happens to the glucose which disappears immediately from the blood when insulin is given to an intact animal. Cori, who with his colleagues have conducted many such balance studies, has pointed out the technical difficulties of this approach (11). The principal difficulty is the relatively small amount of blood glucose compared with the amount of this sugar present in the form of glycogen of muscle and other organs. In order to show an effect of insulin on deposition of muscle glycogen, relatively large amounts of glucose had to be given simultaneously with the insulin, and experiments had to be prolonged considerably beyond the time when insulin exerts its maximal hypoglycemic effect.

However, use of C^{14}-labeled glucose avoids the limitations imposed by balance studies. By labeling the blood glucose, its translocation into tissues and its conversion to metabolic products can be followed with

* Aided by funds provided by the National Cancer Institute, National Institutes of Health, the American Cancer Society, and the United States Atomic Energy Commission, contract No. AT(30-1)777.

† This work will constitute part of a thesis to be presented to the Graduate Council of Temple University by Herman W. Levin in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

relative ease, even though the quantities transformed are too small to be detected by quantitative analysis. In the present study, this procedure was used to determine early effects of insulin administration on glucose oxidation and on the conversion of glucose to liver and muscle glycogen in normal, fasted, and post-absorptive rats.

Methods and Results

Conversion of Glucose to Respiratory CO_2—Our first attempts were directed to demonstrating an early effect of insulin on glucose oxidation in the intact rat. Pairs of male rats from our stock colony were chosen for uniformity of age and weight and were fasted from 20 to 24 hours. They were then given a trace dose of uniformly labeled glucose by intraperitoneal injection. One rat was given, at the same time, an intraperitoneal injection of insulin in a dosage of 20 units per kilo, a quantity sufficient to produce a rapid hypoglycemia. The other rat was used as a control. Each was immediately placed in a glass metabolism chamber, and respiratory CO_2 was collected quantitatively at 15 minute intervals as previously described (12). The carbonate which collected in the bead tower was precipitated as $BaCO_3$, which was then filtered, dried, weighed, and assayed for radioactivity. Fasted rats do not tolerate insulin well in the dosage given. After 90 to 120 minutes they begin to have convulsions and die in about 3 hours. This was not regarded as a serious obstacle to our purpose, since we were primarily interested in only short periods following insulin administration. However, it prevented comparisons over extended periods.

The results proved disappointing, since the differences observed were minimal. Insulin did not cause a measurable change in total CO_2 output, but in favorable experiments the specific activity of the CO_2 rose more rapidly and reached a higher peak in the insulinized than in the control rats. In general, results of these experiments indicated an immediate acceleration of glucose oxidation by insulin, but the differences were not sufficiently clear-cut to be conclusive. It is evident from the work of other investigators (13) that there is a lag period of the order of 30 minutes or longer between the intracellular appearance of CO_2 and its collection from a respiration chamber, owing to the relatively large dilution by the "pool" of bicarbonate in the blood. Contributing also to the lag is the time necessary for absorption of the CO_2 from the metabolism chamber. It was felt that this could be minimized (though not entirely eliminated) by direct collection and assay of the bicarbonate in the blood. This procedure had the further advantage that it could be combined with the isolation and assay of tissue glycogen. After considerable trial, the following satisfactory procedure was developed.

For purposes of illustrating the procedure, and the data which can be calculated therefrom, a typical experiment is described in detail below and the data are given in Table I. Two male rats, matched for age (about 3 months) and weight (about 200 gm.), were each given an intraperitoneal injection of 1 ml. of an aqueous solution of 0.1 mg. (1.1 μc.) of uniformly labeled glucose. One was given in addition a solution of glucagon-free insulin[1] at a dosage of 20 units per kilo (approximately 4 units), while the other received the same amount of buffer solution without insulin.

TABLE I

Blood Bicarbonate and Muscle and Liver Glycogen after Glucose-C^{14} Injection

	Glucose metabolism products					
	Insulinized			Control		
	Blood bicarbonate	Liver glycogen	Muscle glycogen	Blood bicarbonate	Liver glycogen	Muscle glycogen
Size of sample taken	3*	5.26†	21.2†	3*	5.00†	21.3†
Amount of product isolated, mg.	10.5‡	1.1	68.5	10.2‡	1.1	55.9
Specific activity§	374	70	33	263	63	3
Total activity‖	836	3	370	567	3	25
Per cent incorporated¶	1.8	0.006	0.8	1.3	0.005	0.05

* In ml.

† In gm.

‡ As $BaCO_3$.

§ Specific activity = counts per minute per standard planchet, corrected for self-absorption.

‖ Total activity = specific activity × milliatoms of carbon in sample.

¶ Per cent of original dose incorporated = (total counts incorporated × 100)/-total counts administered.

Exactly 15 minutes later each rat was decapitated with heavy shears, and the blood was collected in a small beaker containing a few drops of heparin solution. Exactly 3 ml. were immediately pipetted into a 25 × 3 cm. test tube containing 5 ml. of 0.5 M sodium hydroxide, 0.25 mmole of sodium carbonate as a carrier, and a few drops of silicone antifoaming agent. The tube was tightly stoppered and kept at room temperature for subsequent evolution and collection of the blood CO_2. Another 0.2 ml. portion of blood was deproteinized by the method of Somogyi (14) for blood sugar determination by the anthrone method (15). Meanwhile, the liver was dissected as quickly as possible, weighed, and placed in hot 30 per cent KOH for isolation of glycogen by the method of Boxer and

[1] Kindly supplied by Eli Lilly and Company, Indianapolis, Indiana.

Stetten (16). The same was then done with the muscle, all of the easily obtainable skeletal musculature being included. Approximately 5 gm. of liver and 20 gm. of muscle were worked up in these experiments, the former being placed in alkali within 3 minutes, and the muscle within 10 minutes of the death of the rat.

The CO_2 was liberated from the blood by addition of dilute sulfuric acid and was drawn by suction into a bead tower containing an excess of CO_2-free NaOH solution, by means of a stream of CO_2-free air.

The glycogen was purified by repeated precipitation from a 10 per cent trichloroacetic acid solution with 1.2 volumes of alcohol and, after washing with 95 per cent ethanol, was precipitated from a water solution and dried in a vacuum desiccator. The glycogen was weighed, and a sample was oxidized quantitatively by persulfate (17) to CO_2 for radioactivity assay. This procedure yielded the theoretical amount of CO_2 from glycogen.

Radioactivity determinations were made with an ultrathin window counting tube with $BaCO_3$ samples spread over planchets of 7.5 sq. cm. area. The specific activities are presented in Table I as counts per minute at "infinite thickness" after correction for self-absorption and for carrier. The glycogen levels were very low, of course, in these 24 hour fasted rats: about 0.02 per cent, with a range of 0.01 to 0.5 per cent in liver, and about 0.3 per cent in muscle, with a narrow range of from 0.2 to 0.4 per cent. No significant differences in liver or muscle glycogen *content* were observed between the control and insulinized rats, nor were significant differences seen in blood bicarbonate content, which ranged from 1.28 to 2.27 mmoles per 100 ml., with an average of 1.69.

As shown in Table I, the specific activity of the blood bicarbonate was raised by insulin injection. In this typical experiment, the blood bicarbonate was diluted with 53.4 mg. of carrier barium carbonate to yield 63.9 mg. total, with an activity of 61.3 c.p.m. On correcting for carrier, the blood CO_2 amounted to 10.5 mg. of $BaCO_3$ and had a specific activity of $63.9 \times 61.3/10.5 = 374$ c.p.m. This value is to be compared with 263 c.p.m. in the control. As will be seen later, the degree of difference varied from experiment to experiment and was never very large, but a higher incorporation of glucose carbon in the blood bicarbonate of insulinized rats was consistently observed in every experiment of 40 minutes duration or less.

By multiplying the specific activity by the milliatoms of carbon represented in the sample taken, we obtain a value of $374 \times 10.5/197 = 19.9$ c.p.m. for the total activity of 3 ml. of blood. By taking two-thirds of the body weight (189 gm.) as a rough approximation of the bicarbonate pool size (13), the total activity of the blood bicarbonate would be $189 \times 2 \times 19.9/(3 \times 3) = 836$ c.p.m. Thus, of the total 46,000 c.p.m.

injected, 836 × 100/46,000 = 1.8 per cent of the radioactivity given was present as blood CO_2. Similarly calculated, 567 c.p.m. or about 1.3 per cent was present as CO_2 in the control rat. This, of course, only represents the amount of glucose carbon present as blood CO_2 and not the total oxidized carbon, since some CO_2 is already exhaled by this time.

Muscle Glycogen—Much more remarkable were the differences in the incorporation of glucose carbon in the muscle glycogen; typical was the 11-fold increase in specific activity of that from the insulinized rat in this experiment (Table I). The specific activity of 33 c.p.m. represents a total activity in the 68.5 mg. of glycogen (2.51 milliatoms of carbon) of 2.51 × 33 = 83 c.p.m. Assuming that the incorporation in the dissected muscles is typical of the total musculature and that the total musculature is one-half of the body weight ((11) p. 181), there would be (83 × 189)/-(2 × 21.2) = 370 c.p.m., or 370 × 100/46000 = 0.80 per cent of the original dose, in the muscle glycogen. Similarly calculated, only 25 c.p.m. were incorporated in the muscle glycogen in the control rat, representing only 0.05 per cent of the administered glucose.

Liver Glycogen—In contrast with that of muscle, liver glycogen had a very low activity, and no appreciable difference was observed in incorporation between the control and insulinized rat. The values are only very rough approximations owing to the negligible quantities of liver glycogen in these fasted rats. Nevertheless, they clearly indicate that under the conditions of these experiments, liver glycogen formation is very low compared with that of muscle.

Temporal Changes; Blood Sugar—After thus giving a detailed account of a typical experiment, results are presented graphically in Fig. 1 for twenty such experiments conducted over periods of time ranging from 5 to 190 minutes. The changes in blood sugar shown in the upper right section were as expected. The control values remained essentially constant at about 80 mg. per 100 ml., while those of the insulinized rats dropped rapidly, reaching a low point of 30 mg. per 100 ml. in 40 minutes.

Blood Bicarbonate—The bar graphs in the lower right section show the changes in total activities with time in the blood bicarbonate. Though the differences between the control and insulinized rats were not remarkably large, a consistent pattern was observed. Up to 40 minutes, higher values were found with the insulinized rats, the maximal differences occurring at 20 minutes. Later, the control rats caught up with and then exceeded the insulinized animals in CO_2 output from labeled glucose. These data show more clearly than did the experiments in which breathed respiratory CO_2 was collected that insulin causes a rapid, but transient, increase in glucose oxidation in the fasted rat, coincident with its maximal hypoglycemic action.

Muscle Glycogen—As shown in the upper left section of Fig. 1, total muscle glycogen was relatively constant. However, a most remarkable effect of insulin was observed on the incorporation of radioactivity therein (lower left section). This was already evident in 5 to 10 minutes, but the greatest differences were observed after 40 minutes; in contrast with the blood bicarbonate large differences were still evident even after 2 hours. The fact that these differences are coincident in time with the lowering of

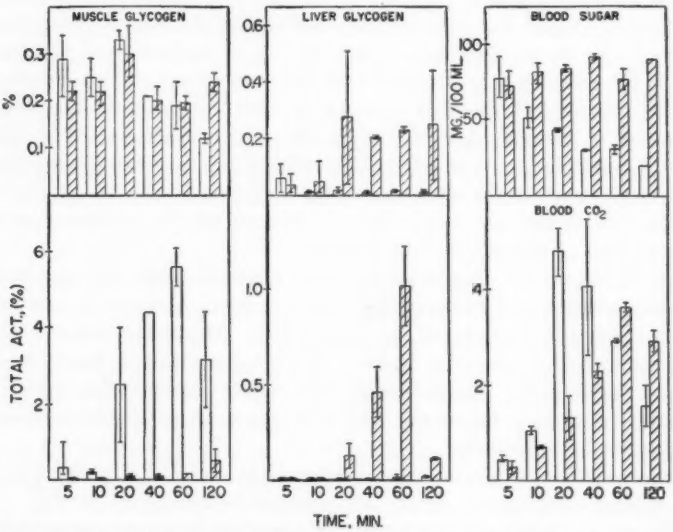

FIG. 1. Effect of insulin on glucose utilization in 24 hour fasted rats. Clear bars are insulinized; lined bars are control rats. Liver and muscle glycogen levels are in per cent of body weight; total activities in per cent of total radioactivity administered as glucose. The values are averages of two, three, or four separate experiments with the ranges as shown.

blood sugar leaves no doubt that insulin brings about an immediate, profound change in the rate of glycogen formation from the circulating blood glucose. It is of interest to note that it would have been difficult, if not impossible, to have observed this effect without labeled carbon. For example in the experiment of Table I, the blood sugar was lowered from 80 to 30 mg. per 100 ml. Assuming a glucose space in the fasting rat of 30 per cent of the body weight, the total transfer of glucose would have been $189 \times 0.3 \times 50/100 = 28$ mg. Since the total muscle glycogen was $0.003 \times 189/2 = 0.29$ gm., even if all of the glucose which disappeared had been converted to muscle glycogen, the increase would only have been about 10

per cent. Actually, the absolute conversion was quite low; even at the peak of incorporation at 60 minutes only about 6 per cent of the total administered glucose was so converted. There was a slow uptake of labeled carbon in the muscle glycogen of the control rats, but this was relatively small. Apparently there is an active turnover, since after 120 minutes the activity declined following the initial rise. However, in one experiment (not shown), even after 190 minutes, the respective specific activities of the insulinized and control rats were 314 and 14 c.p.m.

Liver Glycogen—The liver glycogen levels (upper middle section of Fig. 1) were so low and so variable in these fasted rats that only a crude approximation to its incorporation of the blood glucose could be ascertained. There was a tendency for that of the control rats to increase, and, as seen in the lower middle section, this slight increase in quantity of liver glycogen is accompanied by an increase in turnover. The control rats reached a maximal incorporation in 60 minutes. This was very low, however, in comparison with the muscle glycogen when one notes the large difference in the scales. Even this small incorporation is not shared by the insulinized rats, whose liver glycogen remained extremely low in activity throughout the entire experimental period.

Glucose-Fed Rats—In order to explore further the nature of the action of insulin on liver glycogen, a similar group of experiments was performed with rats given, by stomach tube, a large dose of glucose and which were therefore absorbing glucose during the experimental period. It was reasoned that liver glycogen formation should be rapid under these circumstances and an effect of insulin should be readily demonstrable. Any such effect of insulin should be due to a direct action on the tissue, since Cori ((11) p. 147) demonstrated that insulin has no effect on the intestinal absorption of glucose.

As in the previous experiments, pairs of rats were given trace doses of uniformly labeled glucose-C^{14}, but were also given by stomach tube 5 mmoles of unlabeled glucose in 3 ml. of water. As before, one rat of each pair was given insulin, and the pairs were sacrificed at the intervals shown in Fig. 2. As seen in the upper right square, hyperglycemia ensued in all of the control rats, but those receiving insulin were hypoglycemic to varying degrees at the time of death. As before, CO_2 output from glucose was somewhat greater from the insulinized rats at the shorter intervals, but the differences were not as pronounced as they were in the fasting animals. This result was not unexpected, since the hyperglycemia in the control rats would have been anticipated to elicit endogenous insulin secretion. Total muscle glycogen approximately doubled during the 4 hour experimental period (upper left section) with a slightly greater increase in the insulinized rats. The origin of this increased muscle glycogen from the

injected glucose is evident from the rapid increase in the incorporation of radioactivity, shown in the lower left section. An accelerating effect of insulin was already observed in 10 minutes and, though becoming smaller with time, was maintained throughout the 4 hours. Incorporation reached values greater than 20 per cent of the injected glucose in the insulinized animals, but was very high also in the control rats, in which it reached a peak of 13 per cent of the injected glucose.

As has been already well established, the ingestion of glucose leads to a deposition of liver glycogen (upper middle section). This was slow during

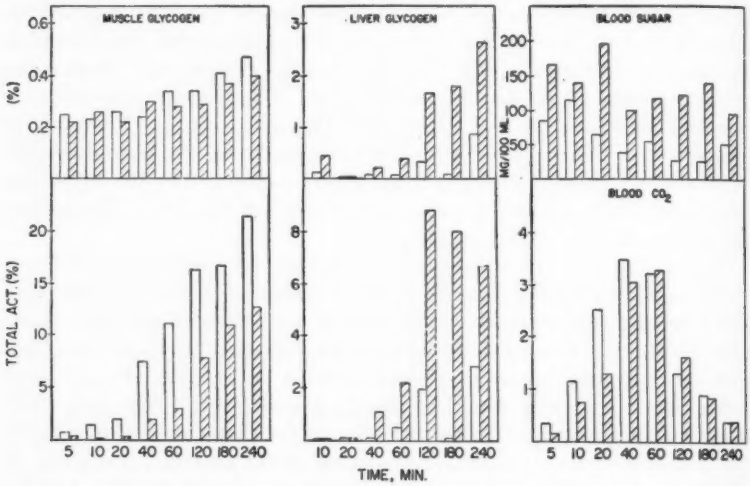

FIG. 2. Effect of insulin on glucose utilization in fed rats. Data calculated as indicated in Fig. 1.

the 1st hour, but increased rapidly thereafter, and at 4 hours the total was well above 2 per cent in the control rats. Liver glycogen deposition occurred also in the insulinized rats, but was much lower than that of the control at every interval. This pattern was observed also for incorporation of radioactivity in liver glycogen (lower middle section), which reached over 8 per cent in the control rats in 2 hours, but only about 2 per cent in the insulinized rats. It is evident that insulin not only does not enhance liver glycogen deposition from ingested glucose, but also actually appears to suppress this process.

Post-Absorptive Rats—As a further test of possible effects of insulin on liver glycogen, it was deemed desirable to learn how insulin would affect its deposition in post-absorptive animals in which the liver glycogen was

already high. Cori (11) has shown that, when rats are given a large dose of glucose, in 4 to 5 hours essentially all has been absorbed from the intestine and that there is a high and uniform liver glycogen content of between 2 and 3 per cent. Experiments conducted in such post-absorptive rats are shown in Fig. 3. As seen in the upper right section, normoglycemia was found in the control and hypoglycemia in the insulinized rats. The same pattern of blood CO_2 activity was found as in the fasted rats. Muscle glycogen content was uniformly high in both control and insulinized

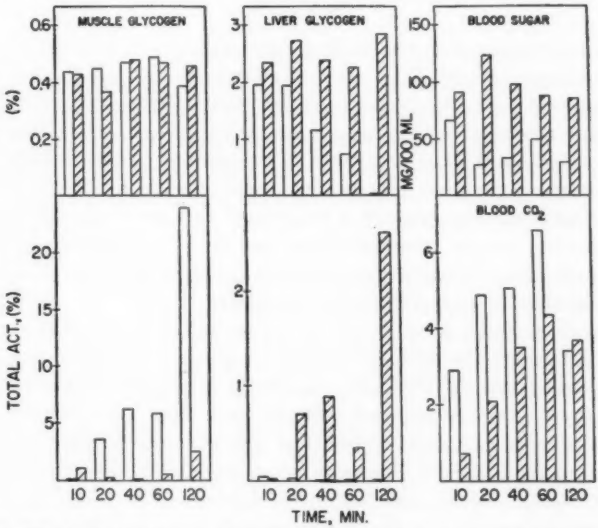

FIG. 3. Effect of insulin on glucose utilization in 4 hour post-absorptive rats. Data calculated as indicated in Fig. 1.

animals, and, again as in the fasted rats, incorporation of glucose carbon therein was extremely high in the insulinzed as compared with the control rats.

As observed previously by Cori (11), liver glycogen was initially at 2 per cent or above in these post-absorptive rats (upper middle section). In control rats the liver glycogen content remained relatively constant at about 2.5 per cent, and during the 2 hours subsequent to labeled glucose administration the radioactivity increased steadily (lower middle section). In contrast, the liver glycogen of the insulinized rats declined steadily and by 2 hours was down to a low fasting level. During this period, there was very little uptake of radioactive glucose (lower middle section). Incorporation of radioactivity was almost negligible at each time interval except the

last, and here, though the specific activity was high, there was so little glycogen present that the total incorporation was actually negligible. These results clearly indicate that there is no immediate uptake of glucose into glycogen of liver, such as occurs in the skeletal muscles. Apparently, much of the uptake in glycogen which would have occurred ordinarily was suppressed by insulin, and even this small amount is further minimized by the secondary glycogenolytic effects which come into play following the hypoglycemia.

DISCUSSION

Many attempts have been made to demonstrate an effect of insulin on glucose oxidation *in vitro*, but, with the exception of the rat diaphragm (18), no unequivocal results have ever been reported. Perhaps the best evidence of a direct effect of insulin on glucose oxidation in skeletal muscle has been provided by Wick *et al.* (19). In eviscerated rabbits, insulin caused a 4- to 5-fold increase in glucose oxidation over an 8 to 9 hour period. However, since the first samples of respiratory CO_2 were taken after a whole hour following insulin administration, and since no differences were apparent at this time, but only after longer intervals, it was uncertain whether this represented an immediate effect of insulin.

It was not until the present work was essentially completed that a report appeared in which the effect of insulin on glucose oxidation was directly tested in intact rats (20) by following the incorporation of glucose carbon in respiratory CO_2. In contrast with our own data from respiratory CO_2 activities, alluded to earlier, Miller *et al.* (20) obtained highly reproducible oxidations of trace doses of glucose in intact rats and found a marked stimulation by insulin, which was evident in $\frac{1}{2}$ hour. Our blood bicarbonate studies, here reported, are in agreement with these results, and together with these show that acceleration of glucose oxidation occurs coincidentally in time with the hypoglycemic action of insulin.

Muscle Glycogen—Abundant evidence is available to indicate that insulin enhances glycogen formation in muscles ((1–6), (11), p. 236, (19, 21)). All of these prior studies were conducted either *in vitro* or over time intervals which were long in relation to the hypoglycemic action of insulin and thus are inconclusive as to whether the observations represent primary effects of the hormone. The results of the present study show that muscle glycogen deposition is enhanced coincidentally in time with the rapid induction of hypoglycemia in normal fasted rats and thus definitely indicate that part of the immediate hypoglycemic action of insulin is in opening a pathway for glycogen synthesis in skeletal muscle.

Liver Glycogen—Definite evidence of an immediate insulin effect on glucose metabolism in liver is scanty and conflicting (21). Numerous studies have shown (22) that impairments of glucose utilization and lipo-

genesis by liver slices of alloxan-diabetic rats are repaired by insulin, but this requires relatively prolonged periods of pretreatment. de Duve and his collaborators (23–25) have championed the view that the primary action of insulin is in accelerating the deposition of liver glycogen. Under the conditions employed here, essentially none of the glucose which disappeared quickly from the blood under the influence of insulin entered the liver glycogen despite the very marked effect in muscle. These observations, if made in the fasting rat only, would have been ambiguous. The liver glycogen in these animals is already depleted. It is conceivable that these small amounts are "turned over" rapidly and a high incorporation may be overlooked through compensatory effects induced by the hypoglycemic condition. However, the same objection cannot be made to those experiments in which glucose was also given. Despite the fact that the control animals repleted their glycogen to approximately 2 per cent in 2 to 4 hours, during this time, by utilizing for this purpose some 6 to 8 per cent of the injected glucose, the insulinized animals raised the liver glycogen level to less than 1 per cent maximum, and the incorporation did not exceed 3 per cent.

The same considerations apply to experiments with the 4 hour post-absorptive rats. These animals had a high liver glycogen initially. If glycogen were being formed but rapidly destroyed, an incorporation of radioactivity would have been observed during the early intervals of this experiment, while the liver glycogen was still high. However, there was negligible incorporation of radioactivity in the insulinized as compared with the control rats, and the only effect of insulin was a depletion of liver glycogen, probably owing to secondary effects of hypoglycemia.

In critically examining all of the available information, Levine and Fritz (21) concluded that insulin does not have a primary action in liver. Similarly, Renold *et al.* (22) assumed that the primary effects of insulin occur in peripheral tissues only, and the apparent effects on liver are due to "metabolic adaptation." The present studies are in agreement with these conclusions in demonstrating that the glucose which leaves the blood under insulin hypoglycemia is not converted appreciably to liver glycogen. Apparently the mechanisms of glucose entry or activation in the liver cells differ from those in the muscle cell; the nature of these differences escapes us at present. These findings do not, however, preclude a direct, immediate effect of insulin in suppressing output of glucose by the liver cells, such as has been observed by Dunn *et al.* (7) and others (8–10).

SUMMARY

Fasted, glucose-fed, and post-absorptive rats were given trace doses of uniformly C^{14}-labeled glucose, with and without the simultaneous intraperitoneal administration of glucagon-free insulin. There was a consistent,

though transient, small rise in the incorporation of radioactivity in the blood bicarbonate and a very marked acceleration of incorporation of radioactivity into skeletal muscle glycogen coincident with the hypoglycemic action in the animals receiving insulin. However, no effect or suppressive action of insulin was observed on the incorporation of glucose carbon into liver glycogen.

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ENZYMATIC OXIDATION OF PYRIDOXAMINE PHOSPHATE TO PYRIDOXAL PHOSPHATE IN RABBIT LIVER*

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(Received for publication, January 9, 1958)

In the course of testing the possible coenzymic function of the phosphorylated vitamin B_6 derivatives on the synthesis of glucosamine 6-phosphate by a rat liver preparation, the appearance of a yellow color similar to that of pyridoxal 5-phosphate was observed in the samples containing pyridoxamine 5-phosphate. Further investigation has revealed the presence of a soluble enzyme system in extracts of several mammalian tissues which catalyzes the oxidation of PAMP[1] to PALP according to the following equation:[2]

$$\text{Pyridoxamine 5-phosphate} + \tfrac{1}{2}O_2 \rightarrow \text{pyridoxal 5-phosphate} + NH_3$$

Detailed studies of the partially purified enzyme from rabbit liver are presented in this report. This enzyme has been shown to be different from the known amine oxidases and also to catalyze the oxidation of pyridoxamine to pyridoxal. The purified oxidase, after partial resolution from its prosthetic group, was reactivated by both flavin adenine dinucleotide and riboflavin 5′-phosphate. In addition, phosphatase activities toward PAMP and PALP were observed in the soluble portion of rabbit liver extracts.

Materials and Methods

Pyridoxamine 5-phosphate, pyridoxal 5-phosphate, and pyridoxamine dihydrochloride were obtained from the California Foundation for Biochemical Research, pyridoxal hydrochloride from the Nutritional Bio-

* This investigation was supported by research grants from the National Institute of Neurological Diseases and Blindness (No. B-141), National Institutes of Health, United States Public Health Service, and the National Council to Combat Blindness, Inc., New York. A preliminary report has been presented (1).

[1] The following abbreviations are employed: PAMP, pyridoxamine 5-phosphate; PALP, pyridoxal 5-phosphate; PAM, pyridoxamine; PAL, pyridoxal; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate. $E^{pH}_{m\mu}$ represents the molar extinction coefficient with a 1 cm. light path.

[2] The balanced equation has been written with the free bases, since the pH optimum is around 10. Presumably, the mechanism of this reaction is by a 2 electron transfer, oxygen-facultative type of reaction (2), but the possible involvement of H_2O_2 as an intermediate has not been determined.

chemicals Corporation, and flavin adenine dinucleotide of 90 per cent purity and riboflavin 5′-phosphate from the Sigma Chemical Company.

Oxidase Assay—The oxidase measurements were performed as follows. PAMP (0.5 μmole per ml.), the enzyme fraction, and 0.1 M Tris buffer of pH 8.0 in a total volume of usually 3.0 ml. were incubated in 25 ml. stoppered Erlenmeyer flasks in a constant temperature shaker at 38° and 70 oscillations per minute. The flasks were then cooled in an ice bath, the contents were transferred to cold small test tubes, and these were placed in a boiling water bath for 2 to 3 minutes. The tubes were cooled, the contents were filtered, and the optical densities were read at 415 mμ in a Beckman model DU spectrophotometer equipped with a microadapter in 1.2 ml. cells of 1 cm. light paths with water as a blank. In later experiments with 0.05 M carbonate buffer at pH 10.4, a final volume of 2.0 ml. was used. The reactions were stopped by the addition of 0.2 ml. of 5 N NaOH, and the optical densities were read at 388 mμ. Only transparent enzyme fractions could be assayed by this procedure, and no precipitate was observed to form upon addition of the base in any of the preparations tested. Some kinetic measurements were also carried out directly in 4.0 ml. cells of 1 cm. light path at room temperature with readings taken at 388 mμ. In these experiments, the blank consisted of the complete reaction mixture minus PAMP. Since PALP is easily decolorized by light, all the procedures were carried out in the minimal amount of light possible.

The molar extinction coefficient of PALP changes markedly with variations in buffer concentration and pH, and the values used for calculations are listed in each experiment. The reported value (3), $E_{388}^{0.1\ M\ NaOH} = 6550$, was used in the experiments with carbonate buffer terminated by addition of NaOH, and values close to this were observed experimentally with commercial PALP. All other values reported were calculated with this value as a measure of absolute concentration.

Specific activities are reported as micromoles of product formed per hour per mg. of protein.

Protein was measured spectrophotometrically (4). In some samples, a small amount of turbidity still remained after the final dilution. These samples were first centrifuged to remove this material, and only the soluble material was measured. Inorganic phosphate was determined by the method of Gomori (5).

Results

The first evidence that this reaction involved a conversion of PAMP to PALP was based upon measurement of the absorption spectrum of the product. A 1.7 to 2.3 M ammonium sulfate fraction of rat liver supernatant fluid (6) was incubated with PAMP in Tris buffer at pH 8.0 at room tem-

perature for 195 minutes. The solutions were then placed in boiling water for 3 minutes and filtered, and the absorption spectra were read. The results (Fig. 1) indicated the appearance of a peak in the complete reaction mixture at 415 mμ which was identical with that obtained with a sample of PALP under the same conditions. The shift of maximum from 388 mμ was the result of the formation of a Schiff base with Tris, as reported in detail by Matsuo (7). Greater enzyme activities were found in rabbit

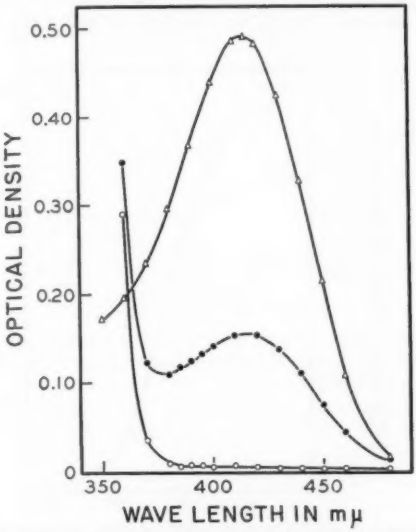

FIG. 1. Absorption spectrum of product formed from pyridoxamine 5-phosphate and rat liver enzyme in Tris buffer at pH 8.0. ●, difference spectrum of complete reaction mixture minus enzyme control; ○, pyridoxamine 5-phosphate (1 μmole per ml.); △, pyridoxal 5-phosphate (0.087 μmole per ml.). The blank was distilled water.

liver than in rat, beef, or calf liver extracts, and it was decided to carry out detailed studies with material from this source.

Partial Purification—A partial purification was accomplished by high speed centrifugation, acid precipitation, alcohol fractionation, and dialysis. All purification procedures were performed at 0–4° unless otherwise indicated. Exsanguinated rabbit livers were placed immediately into cold isotonic KCl. After draining, the liver was homogenized in a Waring blendor for 2 minutes in 2 ml. of cold isotonic KCl per gm. of tissue. The homogenate was centrifuged for 100 minutes at 18,000 × *g*, and the precipitate was discarded. The supernatant fluid was adjusted to pH 5.0 by dropwise addition of 1.0 N acetic acid, and the precipitate was removed by

centrifugation after at least 15 minutes of stirring. 0.3 of a volume of 95 per cent ethanol (cooled to −23°) was added dropwise with constant stirring to this supernatant fluid at 0° placed in a deep freeze at −23°. The precipitate was collected by centrifugation at −2° to −3°, suspended in water, and dialyzed overnight against distilled water. The dialyzed material was centrifuged at 18,000 × *g*, and the insoluble matter discarded. In one such purification, begun with 240 ml. of 18,000 × *g* supernatant fluid, a final preparation was obtained with a specific activity of 0.063 in carbonate buffer and an over-all recovery of 52 per cent.

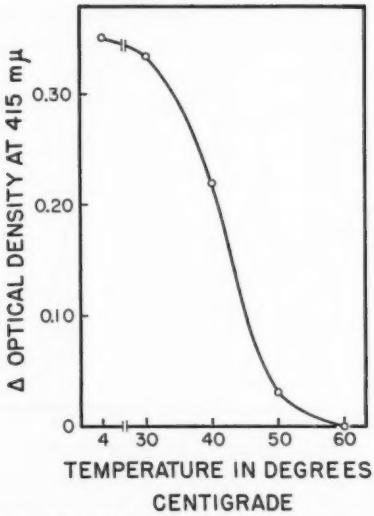

FIG. 2. Heat stability of pyridoxamine-5-phosphate oxidase. 3 ml. samples of the supernatant fluid at pH 5 were incubated for 5 minutes at the indicated temperatures and cooled in an ice bath. Aliquots were assayed for oxidase activity in Tris buffer (pH 8.0) for 60 minutes.

Properties of Enzyme—Specific activities of 0.0040 and 0.0045 were found for two initial 18,000 × *g* supernatant fluids when they were assayed in Tris buffer at pH 8.0 ($E_{415}^{\text{pH 8,Tris}} = 6480$). The partially purified enzyme was stable when stored either frozen or lyophilized at −20°.

The inactivation of this enzyme as a function of temperature is shown in Fig. 2. Heating to 60° for 5 minutes or placing in boiling water for 3 minutes completely destroyed the activity. Linearity of product formation with time was observed at room temperature in phosphate buffer of pH 7.4, in Tris buffer of pH 7.9, and in carbonate buffer of pH 10.4, but

not at 38° and pH 10.4 (Fig. 3). Presumably there is more inactivation under these latter conditions. The effect of varying enzyme concentration on PAMP oxidation is shown in Table I. At both 38° and room tempera-

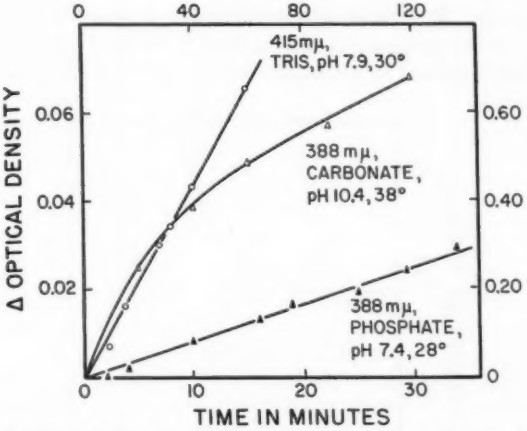

FIG. 3. Oxidase activity of purified enzyme as a function of time. The reactions at 28° and 30° were carried out in cuvettes without shaking (plotted against left-hand and lower scales). The reaction at 38° in 25 ml. Erlenmeyer flasks was carried out with shaking at 70 oscillations per minute (plotted against right-hand and upper scales). Enzyme protein concentrations tested: 28°, 0.89 mg. per ml.; 30° and 38°, 2.7 mg. per ml.

TABLE I

Effect of Enzyme Concentration on Rate of Pyridoxamine 5-Phosphate Oxidation

The purified enzyme and substrate were incubated for 30 minutes at 38° in carbonate buffer (pH 10.4), and the product was determined as described under "Materials and methods."

Enzyme	Pyridoxal 5-phosphate formed
mg. protein	*μmole*
0	0
2.66	0.064
5.32	0.113
7.98	0.140
10.64	0.164

ture, there was a decrease in specific activity as the protein concentration was increased. The pH optimum of this reaction is unusual. In Tris-maleate, Tris, and borate buffers, the activity rises as the pH is raised from

6 to 8, and a plateau is reached. A further increase in pH causes a rise in activity, however, and there is a maximum at pH 10.1 to 10.4 (Fig. 4). Identical results were observed with both a 1 and a 2 hour incubation period. The effect of substrate concentration on the rate of the reaction is shown in Fig. 5. Saturation was reached at 0.2 μmole per ml. of PAMP both at pH 10.4 in carbonate buffer and 9.4 in borate buffer. Only 10 per cent inhibition occurred at 2.0 μmoles per ml. at pH 10.4.

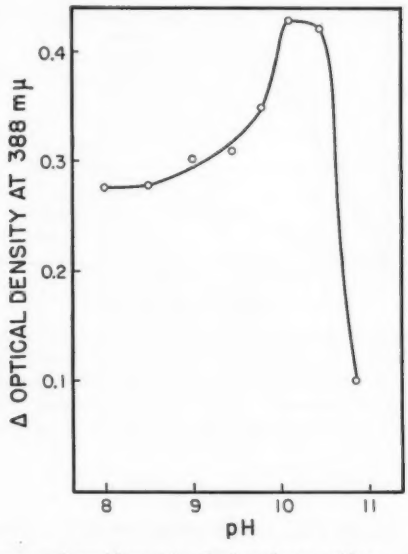

FIG. 4. pH optimum of pyridoxamine-5-phosphate oxidase. PAMP (0.47 μmole) and purified enzyme (5.3 mg.) were incubated in a volume of 2.2 ml. in borate-carbonate buffer (0.05 M) at the final pH values indicated for 1 hour at 38°. The reactions were terminated by the addition of 0.2 ml. of 5 N NaOH.

Establishment of Reaction As Oxidation—Since no substrate in solution other than PAMP was required for the reaction to proceed and there was a very high recovery (>90 per cent) of enzyme activity in the purification procedure, it seemed likely that the enzyme was utilizing oxygen to deaminate the substrate. Direct measurements of oxygen consumption were not possible by conventional manometric procedures because of the low activity of the enzyme, but the evidence in Table II establishes the requirement of oxygen for this reaction to proceed. The reaction was carried out both anaerobically and aerobically in Thunberg tubes, and the amount of product formation was measured spectrophotometrically. It

may be seen that the extent of PALP formation was markedly lowered anaerobically, in one experiment to 2 per cent of that found aerobically.

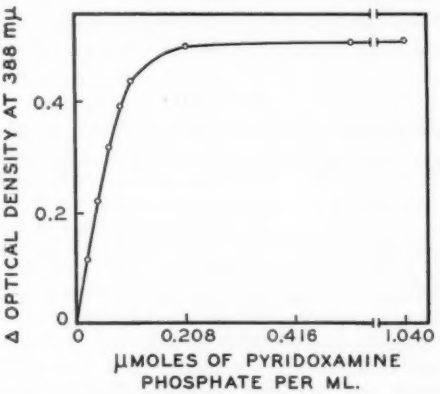

FIG. 5. Effect of pyridoxamine 5-phosphate concentration on oxidase activity. 1 hour incubation in carbonate buffer (pH 10.4) at 38° in 2.0 ml. volume. 0.2 ml. of 5 N NaOH was added to terminate reactions.

TABLE II

Demonstration of Oxygen Requirement for Pyridoxamine-5-phosphate Oxidase

In Experiment 1, the complete cold reaction mixture (3.0 ml.) of pyridoxamine 5-phosphate (1.5 μmoles), purified enzyme, and Tris buffer (pH 7.9) in the main arm of Thunberg tubes was evacuated under 30 mm. of Hg for 3 minutes, with gentle tapping at room temperature. Air was readmitted to one set. The conditions in Experiment 2 were identical, except that the substrate (1.2 μmoles) was placed in the side arm and added to the reaction mixture after evacuation. All tubes were then incubated at 38° with occasional shaking and analyzed as described under "Materials and methods."

Experiment No.	Time of incubation	Δ optical density at 415 mμ		Ratio, $\frac{\text{anaerobic}}{\text{aerobic}}$
		Anaerobic	Aerobic	
	min.			
1	82	0.040	0.316	0.13
	82	0.055	0.327	0.17
2	295	0.021	1.132	0.02
	295	0.326	1.111	0.29

Residual oxygen probably accounts for the small amounts of activity observed anaerobically. Further tests of adding α-ketoglutarate and oxalacetate to PAMP and the enzyme at pH 10.4 revealed 29 and 31 per cent

inhibition, respectively, at 3.3 μmoles per ml. and a slight or no inhibition at 0.33 μmole per ml., but there was no activation in any case.

The measurements of the ammonia formed during this reaction and the stoichiometry of the oxidation are shown in Table III. For each molecule of PAMP utilized, 1 molecule each of PALP and ammonia was formed.

TABLE III

Stoichiometry of Pyridoxamine 5-Phosphate Oxidation

Experiment 1 was run in carbonate buffer (pH 10.4) at 38°, and the reaction was terminated by addition of NaOH. The disappearance of pyridoxamine 5-phosphate was followed by measurement of the decrease in optical density at 310 mμ. Under the above experimental conditions, the following extinction coefficients were found: E_{310} = 8800 for PAMP and 954 for PALP. Thus (optical density (310 mμ))/(8.80 − 0.95) = micromoles per ml. of substrate utilized. The values of PAMP obtained in this manner were identical to those in which the PALP corrections at 310 mμ were calculated by the 388 mμ readings. Experiment 2 was carried out in phosphate buffer of pH 7.0. Samples were placed in boiling water for 2 minutes, cooled, and filtered, and the optical densities of the filtrates were read at 388 mμ. Pyridoxal 5-phosphate formation was calculated, assuming $E_{388}^{pH\ 7.0}$ = 4900 (3). Ammonia was measured in boiled aliquots without filtration by a modified Conway diffusion procedure (8). (These determinations were kindly performed by Mrs. Sheila Shear and Dr. Samuel P. Bessman of the Department of Pediatrics, University of Maryland Medical School, Baltimore.)

Experiment No.	Time of incubation	Pyridoxamine 5-phosphate	Pyridoxal 5-phosphate	NH_3
	min.	*μmole per ml.*	*μmole per ml.*	*μmole per ml.*
1	15	−0.033	+0.032	
	30	−0.052	+0.050	
2	40		+0.026	+0.033
	80		+0.047	+0.048
	150		+0.054	+0.073

The low PALP value in the 150 minute incubation of Experiment 2 probably was caused by the presence in the purified enzyme of a phosphatase which catalyzes the slow disappearance of PALP as measured by 388 mμ absorption (Table VII).

Identity of Product—The identity of the product was further established as PALP by measurement of the activation of tyrosine apodecarboxylase from *Streptococcus faecalis* cells grown on a vitamin B_6-deficient medium (9). PALP is required as a specific coenzyme for this reaction. The results of measurements by this procedure (Table IV) showed close agreement with those found spectrophotometrically. That the PALP formed was not tightly bound to protein, as for example in transaminases, was tested by the following experiment. A series of Warburg flasks containing vitamin B_6-deficient *S. faecalis* cells and tyrosine was incubated at 28° and

pH 5.5, and the liberation of CO_2 was measured manometrically. The combined effect of PAMP and liver oxidase on the rate of the decarboxylation was tested, and the results obtained are shown in Fig. 6. There was a slow rate of tyrosine decarboxylation by the bacterial cells alone, but addition of 0.08 μmole of PAMP at 35 minutes did not increase this rate. Further addition of the oxidase at 65 minutes, however, caused an increasing rate of CO_2 liberation as more PALP was formed. Over a longer period of time, there was also an increased rate of decarboxylation in the control flasks containing PAMP but no liver oxidase. This increase was

TABLE IV

Identification of Product of Reaction As Pyridoxal 5-Phosphate Spectrophotometrically and by Activation of Tyrosine Apodecarboxylase

Pyridoxamine 5-phosphate (0.5 μmole per ml.) and the purified enzyme were incubated at 38° in 0.05 M borate buffer (pH 9.4). At timed intervals, 1 ml. aliquots were removed, 2 ml. of 0.5 M phosphate buffer (pH 7.0) were added, and the tubes were placed in boiling water for 2 minutes, cooled, and filtered. The optical densities were read at 388 mμ, and the results in Column A were calculated by use of $E^{pH\,7.0}_{388} = 0.77 \times 4900$. The borate buffer caused a lowering of the absorption under these conditions to 0.77 of that observed in phosphate buffer. These samples were further tested for activation of tyrosine apodecarboxylase (9), and these results are listed in Column B.

Time of incubation	Pyridoxal 5-phosphate formed	
	A	B
min.	*μmole per ml.*	*μmole per ml.*
0	0.000	0.001
40	0.026	0.032
80	0.037	0.045
120	0.044	0.054

probably caused by the presence of a similar oxidase in *S. faecalis*, and such activity was later demonstrated with whole cells (see Table VI). The occurrence of an increased rate of decarboxylation in the flasks containing bacterial cells and liver oxidase but no PAMP also indicated that these preparations alone still could form small amounts of PALP. This experiment also illustrates a possible physiological role of the PAMP oxidase as a means of activating enzymes which specifically require PALP.

Inhibitors—Azide ion at 0.0034 M did not inhibit the oxidase when it was tested in Tris buffer at pH 7.9. It was not possible to test for inhibition by cyanide ion, since there was a chemical reaction between pyridoxal phosphate and cyanide when they were tested in Tris buffer, which resulted in complete disappearance of the characteristic yellow color of the former; presumably this was through cyanohydrin formation (10).

The remaining compounds were tested for inhibition at pH 10.4 with the partially purified enzyme. Pyridoxamine at 0.005 M showed 34 per cent inhibition but also was deaminated by the enzyme preparation. NH_4Cl inhibited the reaction 15 per cent at 0.0025 M and 34 per cent at 0.01 M. *p*-Chloromercuribenzoate inhibited the oxidase 27 per cent at 0.0001 M

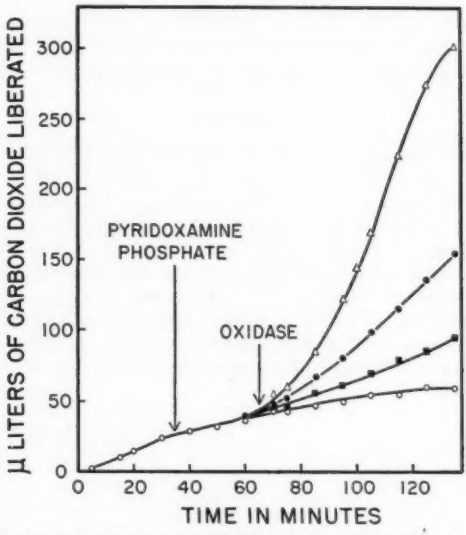

FIG. 6. Activation of tyrosine apodecarboxylase of *S. faecalis* cells (vitamin B_6-deficient) by action of liver oxidase on pyridoxamine 5-phosphate. Warburg flasks containing 0.5 ml. of 0.03 M tyrosine and 6 mg. of *S. faecalis* cells in 1.7 ml. of acetate buffer (pH 5.5) were equilibrated for 20 minutes at 28° before readings were started. After 35 minutes, 0.4 ml. of pyridoxamine 5-phosphate (0.08 μmole) was added from the right side arm, and, after 65 minutes, 0.4 ml. of the purified liver oxidase (6.0 mg.) was added from the left side arm. In the controls, water replaced these materials in the side arms. ○, *S. faecalis* cells alone; ●, cells plus pyridoxamine 5-phosphate; ■, cells plus liver enzyme; △, cells plus pyridoxamine 5-phosphate and liver enzyme.

and 83 per cent at 0.001 M, probably by reacting with sulfhydryl groups. In a kinetic experiment at room temperature, there was a lag period of 5 minutes before measurable inhibition at 0.0007 M. There was no inhibition by pyridoxine or pyridoxal at 0.0002 M, and 26 per cent inhibition by pyridoxine at 0.0033 M. Similar inhibitions were found with oxalacetate and α-ketoglutarate at the latter concentration. $MgSO_4$ had no effect on the oxidase in the concentration range of 0.0005 to 0.05 M.

Nature of Prosthetic Group of Oxidase—The following modification of

the procedure of Warburg and Christian (11) was used to resolve the enzyme from its prosthetic group. Saturated ammonium sulfate was adjusted by the addition of concentrated HCl so that a 1:20 dilution read pH 3.3. All solutions were at 4°, and the pH meter was set for readings at 10°. 4.0 ml. of the acidic ammonium sulfate were added dropwise with constant stirring to 4.0 ml. of the purified enzyme (30 mg. per ml.). The precipitate was immediately collected by centrifugation, washed twice with a mixture of 4 ml. of 0.5 saturated and 1 ml. of saturated ammonium sulfate, resuspended in 3 ml. of distilled water, and dialyzed for 1 hour at 4°. Oxidase activity was then tested alone and with the addition of flavin adenine dinucleotide and riboflavin 5′-phosphate. The results (Table V) indicate partial resolution of the enzyme and reactivation by both

TABLE V

Reactivation of Acid Ammonium Sulfate-Precipitated Oxidase by Flavin Compounds

2 ml. volumes were incubated for 94 minutes at 38° in carbonate buffer (pH 10.4). Reactions were terminated by addition of NaOH. Flavin concentrations were determined by their absorption at 450 mμ by use of reported extinction coefficients (12).

Addition	Pyridoxal 5-phosphate formed
	μmole per ml.
Enzyme alone	0.025
Plus flavin adenine dinucleotide (0.77×10^{-5} M)	0.056
" " " " (1.54×10^{-5} ")	0.063
" riboflavin 5′-phosphate (1.05×10^{-5} ")	0.054
" " " (2.10×10^{-5} ")	0.053

of these flavin compounds. Controls showed no change or slight decreases in absorption at 388 mμ upon treating (*a*) PAMP and the flavins, (*b*) enzyme and the flavins, or (*c*) PALP, NH_4Cl, and the flavins under identical incubation conditions.

Specificity of Oxidase—The question remained as to whether this was a specific oxidase or merely a non-specific conversion by one of the general amine oxidases. No measurable monoamine oxidase activity with the purified liver enzyme was found at pH 7.4 with the spectrophotometric procedure of Tabor, Tabor, and Rosenthal (13) for detecting benzylamine oxidation. Furthermore, the monoamine oxidase activity of liver is in the particulate fraction (14), whereas the PAMP oxidase is in the supernatant fluid. Zeller *et al.* (15) have previously reported that purified preparations of diamine oxidase did not oxidize PAMP or PAM, and there was no measurable PALP formation with a preparation of this enzyme (kindly provided by Dr. Zeller) at pH 7.0 or 7.4 in phosphate buffer under

conditions by which cadaverine was oxidized. There was also no measurable histamine oxidation in phosphate buffer at pH 7.4 by the purified PAMP oxidase, as measured by the colorimetric procedure of Rosenthal and Tabor (16). Differences in pH optima and substrate concentrations that give maximal activity serve further to distinguish this enzyme from diamine oxidase. Dr. Jerard Hurwitz (personal communication) also found no oxidation of PAMP by a partially purified D-amino acid oxidase.

Oxidation of Pyridoxamine—The purified enzyme also catalyzed the oxidation of pyridoxamine to pyridoxal. The concentration of PAM needed for maximal activity was much higher (about 7.5 μmoles per ml.), but the rates of oxidation were of the same order of magnitude. Incubation of PAM (7.5 μmoles per ml.) with the oxidase at pH 10.4 and 38° gave 0.024 and 0.042 μmole per ml. of PAL in 15 and 30 minutes, respectively, under conditions by which 0.035 and 0.055 μmole per ml. of PALP was formed from PAMP present at 0.5 μmole per ml. The absorption of PAL at 388 mμ is not stable at pH 10.4, and these calculations were made on the assumption that $E_{388}^{\text{pH } 10.4} = 2030$. This was the minimal value found with two different solutions of pyridoxal hydrochloride under identical conditions. The product of PAM oxidation was further established as pyridoxal by measurement of tyrosine apodecarboxylase activation after preincubation of the *S. faecalis* cells with adenosine triphosphate (9).

Occurrence of Oxidase in Mammalian Tissues and Microorganisms—The relative activities of PAMP oxidase from several different sources are summarized in Table VI. The largest concentrations were found in liver, and smaller amounts were present in kidney and brain. Negligible activity was observed in rabbit muscle, lung, and heart, and in pig heart. When whole cells of bakers' yeast and *S. faecalis* (vitamin B_6-deficient) were tested directly, small amounts of oxidase activity were found.

Demonstration of PAMP and PALP Phosphatase Activity—In experiments to test the reversibility of the PAMP oxidase reaction, starting with PALP and the purified enzyme, a slow disappearance of 388 mμ absorbance was noted in the absence of added NH_4^+. It was also observed that, when the oxidation of small amounts of PAMP was followed by measurement of 388 mμ absorption, the rate of color formation slowed down and a slow disappearance of 388 mμ absorption took place. These results could have been caused by PALP phosphatase action, since the molar extinction coefficient at this wave length for PAL is much lower than that for PALP. Direct measurement showed the presence of a soluble phosphatase activity toward PAMP and PALP in the high speed supernatant fluid and the purified oxidase fraction at pH 10.4 (Table VII). The specific activity toward PALP was 0.025 with the purified oxidase of specific activity of 0.063. Since the phosphatase activity on PAMP in the 18,000 × *g* su-

pernatant fluid was much higher than the oxidase activity (3.45 μmoles per hour per ml. of enzyme as compared to 0.67), phosphatase activity probably occurs for both compounds.

The rate of PALP disappearance at pH 10.4 in the presence of thoroughly

TABLE VI

Pyridoxamine-5-phosphate Oxidase Activity of Mammalian Tissues and Microorganisms

The results are expressed as micromoles of product formed per hour per gm. of wet weight of tissue. Supernatant fluids of isotonic KCl homogenates were analyzed after 90 minutes of centrifugation at 18,000 × *g*. Group 1: Tris buffer, pH 8.0, $E_{415}^{pH\ 8,\ Tris} = 6480$ for PALP; Groups 2 and 3, carbonate buffer, pH 10.4.

Group No.	Tissue	Activity	Group No.	Tissue	Activity
1	Rabbit liver	0.62–1.10	3	Rabbit liver	2.00
	" kidney	0.22		Rat "	1.13
	Rat liver	0.38		" kidney	0.33
	Beef "	0.12		" brain	0.11
	Calf "	0.12		" lung	0.05
2	Bakers' yeast*	0.26		" heart	0.00
	*S. faecalis**	1.17		" muscle	0.00
				Pig heart†	0.00
				Human serum (per ml.)	<0.01

* Whole cells of dried bakers' yeast and lyophilized *S. faecalis* cells (vitamin B_6-deficient) were analyzed. The results are expressed as micromoles of product formed per hour per gm. of dry weight.

† Homogenized in 0.125 per cent $KHCO_3$.

TABLE VII

Phosphatase Activity of Rabbit Liver Fractions on Pyridoxamine 5-Phosphate and Pyridoxal 5-Phosphate

Substrate, enzyme, and 0.4 ml. of 0.05 M $NaHCO_3$ (pH 10.4) in a final volume of 1.0 ml. were incubated for 2 hours at 38° in stoppered 15 ml. centrifuge tubes. Reactions terminated by addition of 0.5 ml. of 20 per cent trichloroacetic acid

Fraction	Substrate		Inorganic phosphate formed
	PAMP	PALP	
	μmoles per ml.	*μmoles per ml.*	*μmoles per ml.*
18,000 × *g* supernatant fluid (0.2 ml.)	1.5		1.38
		1.5	0.94
Dialyzed alcohol ppt. (0.3 ml., 7.0 mg. protein)	1.5		0.43
		1.5	0.35

dialyzed, purified oxidase, as measured by disappearance of 388 mμ absorption, was found to increase with increasing concentrations of NH_4Cl. In one experiment at 38° with 0.00025 M PALP, the loss of optical density in 41 minutes without added NH_4^+ was 0.062, and this was increased to 0.078 with 0.005 M NH_4^+ and to 0.099 with 0.010 M NH_4^+. The difference in absorption between the samples with and without NH_4^+ approached a constant value after 5 minutes in another experiment at room temperature. This could represent partial reversal to an imide type of intermediate.

DISCUSSION

The known enzymatic transformations of vitamin B_6 are summarized in the accompanying diagram (17). The interconversion of free PAMP and

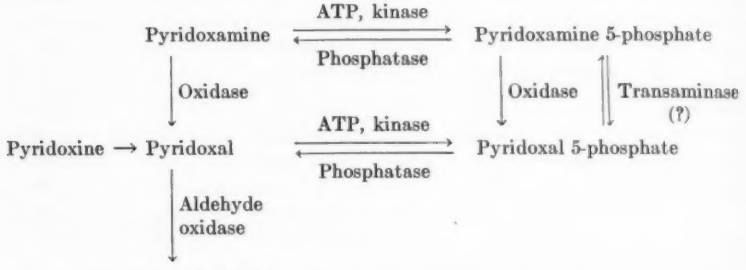

DIAGRAM 1

PALP by transaminase has not yet been demonstrated; however, Jenkins and Sizer (18) recently reported evidence for the formation of enzyme-bound PAMP from glutamate and a highly purified transaminase containing bound PALP. Beechey and Happold (19) also have reported an interconversion of PAMP and PALP by a transamination type reaction, but have not identified the acceptor component of the system. Attempts in this laboratory to show amino acid oxidation by coupling transaminase and PAMP oxidase activities in the presence of appropriate amino acids and PALP were unsuccessful, probably because the protein-bound PAMP is not available for oxidation.

Although pyridoxine, pyridoxamine, and pyridoxal independently can serve as satisfactory dietary sources of this vitamin both for mammals and for many microorganisms (20), each presumably must be converted to PALP, the active coenzymic form of vitamin B_6 for amino acid transamination, decarboxylation, deamination, racemization, and many other reactions (21). Recently, phosphorylase also has been shown to require PALP as a prosthetic group (22). With the exception of transamination,

in which under certain conditions PAMP also is active (23), all of these reactions specifically require PALP. The present oxidase, together with the pyridoxamine kinase described by Hurwitz (24), represents a new pathway for the conversion of PAM to PALP. This transformation also is possible via PAM oxidation and PAL kinase.

PAMP oxidase may play an important role in the control of metabolism, particularly that of amino acids. One example of this was illustrated in the activation of the tyrosine apodecarboxylase in *S. faecalis* cells by the addition of PAMP and the oxidase. This oxidase reaction also may be adaptable as a procedure for the quantitative measurement of PAMP and PAM levels in natural products.

SUMMARY

1. A soluble enzyme system has been partially purified from rabbit liver extracts which catalyzes the following reactions:

$$\text{Pyridoxamine 5-phosphate} + \tfrac{1}{2}O_2 \rightarrow \text{pyridoxal 5-phosphate} + NH_3$$
$$\text{Pyridoxamine} + \tfrac{1}{2}O_2 \rightarrow \text{pyridoxal} + NH_3$$

2. Studies on the properties and specificity of this system are presented. The resolved enzyme was reactivated by both flavin adenine dinucleotide and riboflavin 5′-phosphate. Evidence is given to show the dissimilarity of this oxidase from the known amine oxidases.

3. Phosphatase activities toward both pyridoxamine 5-phosphate and pyridoxal 5-phosphate occurred in soluble extracts of rabbit liver.

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THE ALKALINE PHOSPHATASE CONTENT OF HUMAN MILK

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(Received for publication, January 29, 1958)

The possibility that enzyme systems normally present in breast milk may account for the superiority of this food over pasteurized formulas in promoting the health and nutrition of the newborn infant has been the subject of speculation and study. Of the many enzymes present in milk, some attention has been directed to lysozyme (1, 2), lipase (3, 4), and phosphatase (5–7) as substances which may influence the health and nutrition of the newborn infant.

Clinical observations[1] on the feeding of cows milk formulas supplemented with phosphatase indicate that this substance may overcome feeding difficulties of some infants and have led to the present study of the occurrence of alkaline phosphatase in normal breast milk.

The presence and classification of phosphatase enzymes (7) as well as their relationship to the occurrence of phosphorus in various forms (5) have been established for human milk. This work was done on a limited scale and included only the values on milk of the 1st month of lactation.

The present study, in which 199 samples from twenty donors were analyzed, will show that alkaline phosphatase occurs in variable amounts in human milk. Although there appears to be a tendency for phosphatase concentration to be related to the fat content of the milk, there is no apparent correlation with nitrogen content or total solids other than fat. There also seems to be no relationship to age, nationality, or other characteristics of the donor, except that for most of the seven donors from whom samples were taken over a period of 3 or more months a tendency for the phosphatase content of the milk to increase with duration of the lactating period was observed.

EXPERIMENTAL

Selection of Breast Milk Samples—Fresh, unpasteurized human milk samples were obtained by special arrangement with the San Francisco Mothers' Milk Bank. Because milk bank donors are limited to mothers who are successfully nursing their infants, this study can be considered as limited to nutritionally normal human milk.

[1] Dr. L. Breslow, Chicago, Illinois, personal communication.

In all instances, early morning samples were used, some expressed before and some expressed after nursing the infant. Immediately after expression, the samples were placed in an ice bath until chilled, and then transferred to the refrigerator, where they were held for 1 to 2 hours before collection by the milk bank drivers. At the milk bank a 30 ml. sample was transferred to a screw cap culture tube and held under refrigeration until taken to the analytical laboratory. All samples were analyzed within 4 to 6 hours after expression.

During the course of the study it was determined that under these conditions there was no significant loss in phosphatase activity from the time of expression until the samples were analyzed.

Measurement of Alkaline Phosphatase Activity—Several methods are available for the quantitative estimation of alkaline and acid phosphatase in biological materials (8–21). In all techniques a phosphoric acid ester serves as the substrate, and colorimetric determinations are made on either the inorganic phosphate or the organic moiety of the substrate released by enzymatic hydrolysis. Each of these methods was investigated and a modified procedure based on the Scharer phosphatase test (20) was selected for the following reasons: (1), the rate of hydrolysis of the substrate, disodium phenyl phosphate, is linear for a period of 1 hour; (2), the hydrolysis product, phenol, is readily measured by combining it with the Gibbs reagent (22), 2,6-dibromoquinonechlorimide, to form a blue-green indophenol dye; (3), this indophenol dye can be extracted from milk in a clear solution with *n*-butyl alcohol; (4), reproducible results are readily obtained.

Methods in which *p*-nitrophenyl phosphate (12–15) was employed were investigated and, although the hydrolysis product, *p*-nitrophenol, is a self-indicator, the color could not be extracted into a clear solution for colorimetric measurement.

The Huggins and Tallalay method (11) in which phenolphthalein is the hydrolysis product did not lend itself to the estimation of phosphatase activity in milk because the rate of hydrolysis was not linear and the color could not be clarified by extraction. The Bodansky method (8) was attempted but not used because it was found that phenol could be measured more readily than inorganic phosphate.

The analytical procedure finally adopted for the estimation of alkaline phosphatase in human milk was as follows: (1) Dilute 5 ml. of milk and 10 ml. of 5 per cent $MgSO_4 \cdot 7H_2O$ solution to 100 ml. with distilled water to make the test solution; (2) prepare the buffered substrate by mixing 6 ml. of 1 M Na_2CO_3 with 4 ml. of 1 M $NaHCO_3$ and 10 ml. of 0.02 M disodium phenyl phosphate and dilute to 100 ml. with distilled water; (this buffered substrate has a pH of 9.5 at 37.5°); (3) incubate 1.0 ml. of the

test solution with 5.0 ml. of buffered substrate at 37.5° for 1 hour in a test tube; (4) place the tube in boiling water for 5 minutes and then cool the contents to 37.5°; (5) add 0.5 ml. of 0.2 per cent 2,6-dibromoquinone-chlorimide in absolute methanol to the tube, mix, and allow to stand for 15 minutes; (6) extract the blue-green color developed by adding 10 ml. of *n*-butyl alcohol to the tube, with thorough mixing and centrifuging; and (7) decant a clear *n*-butyl alcohol layer into a cuvette and measure the optical density at 660 mμ.

A blank was run for each sample by carrying 1.0 ml. of test solution, inactivated by holding in boiling water 5 minutes, through the entire procedure.

The phosphatase activity was determined as the micrograms of phenol liberated under the conditions of the test and was calculated by using an internal standard in which 10 γ of phenol are treated in the procedure. The following calculation was used: phosphatase activity per ml. of milk = (optical density of test solution × 200)/(optical density of 10 γ of standard).

Several factors influenced the results of the test and precautions were taken to incorporate into the procedure those conditions which tended to give maximal phosphatase activity.

Mg^{++} Activation—The use of Mg^{++} increased the phosphatase activity approximately 40 per cent. Folley and Kay (16), Motzok (23), and Roche (24) have reported Mg^{++} as well as other divalent cations as activators for phosphatase. The concentration of 0.003 M Mg^{++} was found to be optimal in the conditions of this test.

pH Optimum—The effect of pH on the phosphatase activity of human milk was tested with use of various buffer systems. The Veronal-acetate buffer system of Michaelis (25) was found to give slightly higher values than the carbonate-bicarbonate buffer system of Delory and King (26) when tested with a variety of milk samples. Both buffer systems showed a pH optimum of 9.4 to 9.5 at 37.5° for alkaline phosphatase. With the Michaelis buffers, a pH optimum of 4.8 to 5.6 at 37.5° was found for the acid phosphatase of human milk. The carbonate-bicarbonate buffer was adopted because of the availability of suitable quality ingredients.

Maximal Absorption—With a Coleman model 14 spectrophotometer being used, maximal adsorption of the *n*-butyl alcohol indolphenol dye solution was found at 660 mμ. This maximal absorption point is in agreement with the values reported by Ettinger and Ruchhoft (22).

Measurement of Other Constituents of Human Milk—Total solids were determined by evaporating 5 ml. samples in aluminum dishes to apparent dryness on a hot plate, followed by drying under 29 inch vacuum at 70° for 4 hours.

Fat was determined on 5 gm. samples by the Roese-Gottlieb method (27).

Nitrogen was evaluated on 5 gm. samples by the Kjeldahl-Wilfarth-Gunning method (28). The NH_3 was collected in 4 per cent boric acid and titrated to the methyl red end point with 0.1 N HCl.

"Solids-not-fat" represents the difference between per cent total solids and per cent fat.

Results

Samples of milk over varying periods of lactation were obtained from a total of twenty donors. One sample was obtained from six available donors every week and determinations of phosphatase, total solids, per cent fat, per cent nitrogen, and per cent solids-not-fat were made. A total of 199 evaluations of alkaline phosphatase was made over a period of 9 months. The highest value found was 540 units per ml. and the lowest value, 30 units per ml. The average of all these evaluations was 147 units per ml.

The average value of 147 determinations of total solids was 11.40 per cent; of 146 determinations of fat, 2.77 per cent; of 116 determinations of nitrogen, 0.157 per cent; and of 146 determinations of solids-not-fat, 8.65 per cent. The age, nationality, number of children, and the average values obtained for phosphatase, total solids, fat, nitrogen and solids-not-fat are found in Table I.

To determine the existence of an upward or downward trend in phosphatase content with duration of lactation, Table II was constructed. Only those donors (seven) from whom samples were taken over a period of at least 3 months were considered. The tremendous variation in average phosphatase content from individual to individual, plus the fact that in this experiment the weekly samples were collected for varying lengths of time, beginning at different intervals post partum, prohibits any over-all correlation between phosphatase content and duration of lactation. However, from inspection of Table II, it appears that several of the individuals do show a tendency to secrete a higher concentration of phosphatase as lactation proceeds. Actual correlations of phosphatase content with weeks of lactation were made for the samples from each of the seven donors. The correlation coefficients are shown in Table II, four of which are statistically significant at a probability level of 0.01 or less.

Inspection of the individual sets of analyses also indicated a possible correlation between phosphatase and fat content. The correlation coefficient calculated for 145 samples which were analyzed for both phosphatase and fat is 0.57, a value which, because of the large number of

observations, is statistically highly significant ($P < 0.001$) despite the wide range in values.

TABLE I

Comparative Levels of Alkaline Phosphatase and Other Constituents of Human Milk

Donor	Age	Nationality	No. of children	Phosphatase*				Per cent total solids		Per cent fat		Per cent nitrogen		Per cent solids-not-fat	
				No. of determinations	Average	High	Low	No. of determinations	Average	No. of determinations	Average	No. of determinations	Average	No. of determinations	Average
A. M.	30	Scotch-Irish	5	3	50	54	41	3	11.54	3	2.82	3	0.168	3	8.65
P. M.	27	Irish	4	8	52	75	33								
L. H.				3	53	57	50	3	11.47	3	2.34	3	0.219	3	9.13
E. E.	33	Norwegian	2	15	57	120	33	7	11.50	7	3.43			7	8.23
D. R.	22	Caucasian	1	3	59	80	48	3	10.22	3	1.47	3	0.150	3	8.75
M. P.	19	Russian	2	5	64	81	48	5	12.07	5	3.31	5	0.187	5	8.76
M. H.				1	67	67	67	1	10.18	1	1.34	1	0.188	1	8.84
C. M.	35	German-English	2	2	73	74	72	2	10.25	2	1.65	2	0.175	2	8.60
L. K.	30	Japanese	2	39	74	180	30	30	10.75	30	2.32	24	0.127	30	8.50
J. B.	20	Polish-German	1	2	88	93	84	2	10.76	2	2.09	2	0.184	2	8.62
J. S.	19	Scotch-Irish-English	1	3	105	146	70	3	12.07	3	3.23	3	0.204	3	8.84
E. A.	26	German-French	1	8	113	157	71	8	10.38	8	1.70	7	0.172	8	8.77
Y. M.	23	French	3	8	115	166	30	8	11.14	8	2.25	8	0.202	8	8.87
Y. B.	24	Caucasian	2	9	145	280	90								
J. P.	33	German	2	4	151	212	89	4	11.88	4	2.70	2	0.221	4	9.16
R. A.	26	Irish	1	3	152	166	134	3	12.60	3	3.63	2	0.182	3	8.94
B. C.	28	Irish-English	1	16	169	400	42	15	10.81	15	2.19	15	0.154	15	8.56
S. V. L.	32	German-English	4	28	240	540	66	20	11.65	20	3.12	15	0.145	20	8.56
V. T.	26	Caucasian	4	37	248	492	93	28	12.37	27	3.74	21	0.145	27	8.74
E. P.	26	"	1	2	329	452	206	2	12.80	2	3.72			2	9.06
Average				199	147			147	11.40	146	2.77	116	0.157	146	8.65

* Phosphatase activity expressed as micrograms of phenol liberated per ml. of milk.

No relationship was discernible between phosphatase content and nitrogen content or solids-not-fat content.

TABLE II

Effect of Duration of Period of Lactation on Average Phosphatase Levels of Human Milk

Lactation	Average alkaline phosphatase level of individual donor						
	Y. M.	E. A.	S. V. L.	B. C.	L. K.	V. T.	E. E.
wks.							
0–5	30 (1)*						
6–10	95 (4)	85 (3)	164 (2)				
11–15	132 (3)	121 (2)	184 (5)	120 (4)			
16–20		135 (3)	303 (5)	127 (5)	91 (3)		
21–25			354 (5)	255 (5)	69 (5)		
26–30			376 (4)	161 (2)	68 (5)		
31–35					57 (4)	286 (1)	
36–40					52 (5)	143 (5)	48 (8)
41–45					59 (3)	303 (5)	55 (4)
46–50					74 (5)	244 (5)	84 (3)
51–55					126 (1)	250 (5)	
56–60						225 (4)	
61–65						178 (4)	
Correlation coefficient...	0.90†	0.81†	0.68‡	0.41	−0.01	−0.01	0.64†

* The numbers in parentheses indicate the number of observations.
† Probability level 0.01.
‡ Probability level 0.001.

DISCUSSION

The outstanding finding in this study is the wide variation in alkaline phosphatase activity of human milk, both from mother to mother and during the period of lactation of a given individual.

The values obtained for alkaline phosphatase did not differ widely from those reported by Chanda and Owen (5), although samples in the present study were mostly from women who had been lactating for 2 months or more.

The values obtained for per cent of total solids, fat, nitrogen, and solids-not-fat compared favorably with the reported data of other investigators (29–32).

The function, if any, of phosphatase in human milk is unknown. Cranston (33) has proposed that it aids in the absorption of carbohydrate by the infant, but the mechanism is obscure. There may be some theoretical importance to the apparent tendency of phosphatase to increase with fat content, but a correlation coefficient, even when it is much higher than in the present instance, does not in itself imply any cause or effect relation-

ship. One could also conjecture that the phosphatase is linked in some manner to a minor constituent of the milk.

There appeared to be no relationship between the phosphatase values and the age or nationality of the mother, or the number of children in her family, though the number of donors was too small to test these hypotheses adequately. The small number of subjects involved and the variability of the results also make it unwise to draw conclusions about variation of phosphatase content with length of lactating period, even though some of the data of this study suggest that a relationship may exist.

SUMMARY

1. A method of measuring alkaline phosphatase activity of human milk is described.

2. Analyses of 199 samples of human milk gave an average alkaline phosphatase level of 147 units per ml. The range was from 30 to 540 units per ml.

3. Some tendency for high phosphatase levels to be associated with a high fat content was observed. Other than this there appeared to be no relationship of phosphatase values to the other constituents examined.

4. Other than a possible tendency for alkaline phosphatase concentration to increase as lactation progressed, no relationship was found between the level of the enzyme and such characteristics of the donor as age, nationality, or the number of children.

The authors wish to thank the Mothers' Milk Bank, Inc., and its sponsor, the San Francisco Branch of the American Association of University Women, for their help and cooperation in making this study possible, and Miss Shirley Brazda and Mrs. Judy Pelts for their technical assistance.

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ASPERGILLIC ACID: AN ANTIBIOTIC SUBSTANCE PRODUCED BY ASPERGILLUS FLAVUS

III. THE STRUCTURE OF HYDROXYASPERGILLIC ACID*

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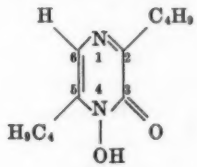
(Received for publication, November 4, 1957)

The elaboration of antibiotic substances by microorganisms is generally accompanied by the production of other antibiotic compounds, which are often structurally related to the main product. *Streptomyces fradiae* produces two closely related substances, neomycin B and neomycin C (1), in addition to an unrelated, antifungal substance, fradicin (2). *Streptomyces aureofaciens* produces both tetracycline and chlorotetracycline (3), and *Streptomyces griseus* makes streptomycin, mannosidostreptomycin, hydroxystreptomycin, actidione, and candicidin (4).

In studying the production of antibiotics by a strain of *Aspergillus flavus*, Menzel, Wintersteiner, and Rake (5) observed that on certain media the predominant product was aspergillic acid (6) while with other media and conditions of growth the product was principally a related substance named hydroxyaspergillic acid. From published reports it appears probable that the antibiotic products formed by other strains of *A. flavus* or related organisms may represent still other variations of the basic structure (7–11).

Because of the more promising biological activity of aspergillic acid the investigation of its chemical structure was undertaken first. Evidence was obtained which permitted the assignment of the structure shown in Formula I to this molecule (6). In the absence of conclusive proof, the two side chains were provisionally assumed to be *sec*-butyl groups. A revision

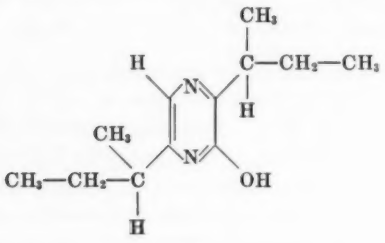


(I)

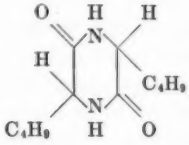
of this structure was indicated, however, in the light of evidence subse-

*A preliminary report of this work was given at the Thirty-ninth meeting of the American Society of Biological Chemists at Atlantic City, March, 1948 (19).

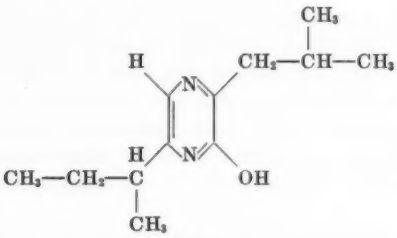
quently furnished by the structural examination of hydroxyaspergillic acid as described in the present paper. A similar conclusion was reached by Newbold and Spring (12) who synthesized racemic 3-hydroxy-2,5-di-*sec*-butylpyrazine (II), the structure which had been postulated for deoxyaspergillic acid, a reduction product of aspergillic acid (6). These workers found that the differences in properties between the synthetic product and that derived from aspergillic acid precluded identity. Dunn and cowork-



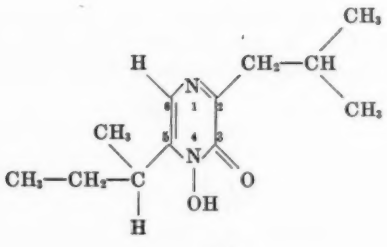
(II)



(III)



(IV)



(V)

ers further compared the diketopiperazine (III) derived from aspergillic acid (6) with various synthetic products and found its closest resemblance was to DL-isoleucyl-DL-leucine anhydride (13). Subsequently it was demonstrated that on vigorous hydrolysis the diketopiperazine (III) yielded a

mixture of DL-leucine and DL-isoleucine (14). Final proof that deoxyaspergillic acid was 3-hydroxy-2-*iso*-butyl-5-*sec*-butylpyrazine (IV) was obtained by comparison of the racemized deoxyaspergillic acid with the synthetic DL compound of known structure (15). The correct structure of aspergillic acid is therefore that shown in Formula V.

This paper, which describes the determination of the structure of hydroxyaspergillic acid, provides additional evidence for the presence in these two antibiotics of both an *iso*-butyl and a *sec*-butyl side chain.

The separation of hydroxyaspergillic acid from aspergillic acid was accomplished by means of their differential solubility in hexane or by countercurrent distribution between ether and 25 per cent aqueous sulfuric acid.

TABLE I

Comparison of Properties of Aspergillic Acid and Hydroxyaspergillic Acid

Property	Aspergillic acid	Hydroxyaspergillic acid
Crystal form and color	Yellow needles from methanol	Nearly colorless needles from hexane
M.p. (uncorrected), °C.	97–99	148–150
Composition	$C_{12}H_{20}N_2O_2$	$C_{12}H_{20}N_2O_3$
Mol. wt.	224	240
Specific rotation, *degrees*	+13.4 (c = 1.0 in ethanol)	+36 (c = 1.0 in ethanol)
pK_a	5.5	4.9
Ultraviolet spectrum	λ_{max} = 328 mμ, ϵ = 8,500 " = 235 " " = 10,500	λ_{max} = 328 mμ, ϵ = 8800 " = 235 " " = 8750
Cupric salt	Green, rectangular plates, m.p. 198–199°	Green, rectangular plates or cubes, m.p. 216°

The properties of the purified compounds are compared in Table I. Elemental analysis showed that the hydroxyaspergillic acid contained 1 oxygen atom more than aspergillic acid. As the name implies, hydroxyaspergillic acid was presumed to be identical with aspergillic acid except for the presence of an additional hydroxyl function, a deduction based on the following observations: (1) the carbon and hydrogen numbers are the same, (2) the ultraviolet absorption spectra are essentially identical, and (3) the acidic function is a hydroxamic acid grouping in both compounds. The observation that hydroxyaspergillic acid did not react with bromine nor couple with diazonium reagents as readily as did aspergillic acid suggested that the additional oxygen atom of the former might occupy the nuclear position 6 in V, but this hypothesis was soon ruled out in view of the results obtained by the treatment of hydroxyaspergillic acid with hydriodic acid in acetic acid solution.

This reaction, in the case of aspergillic acid, had been found to reduce

the hydroxamic acid grouping to the amide, yielding deoxyaspergillic acid (IV). The product obtained by the similar reduction of hydroxyaspergillic acid was a neutral compound, $C_{12}H_{18}N_2O$, in which not only had the hydroxamic group been reduced but the elements of water had been eliminated. Such an elimination is inconsistent with a structure in which the hydroxyl group is located at position 6 of the ring. The hydroxyl group of hydroxyaspergillic acid must therefore be located on one of the butyl side chains. The ultraviolet absorption spectrum (Fig. 1) of the neutral

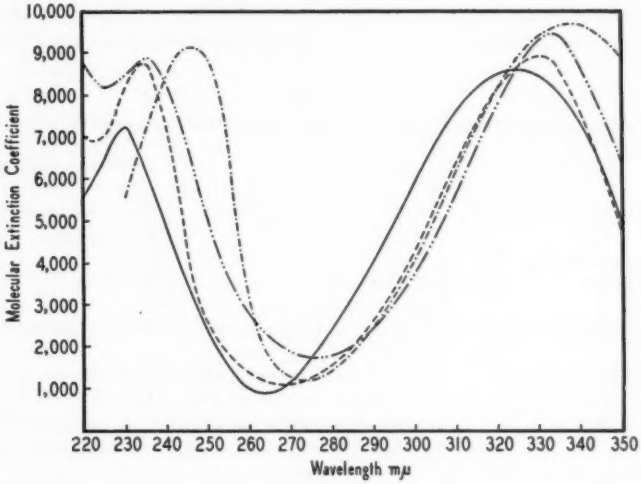


FIG. 1. Ultraviolet absorption spectra. Dashes, hydroxyaspergillic acid; solid line, deoxyaspergillic acid; dash and two dots, dehydroaspergillic acid; dash and one dot, dehydrodeoxyaspergillic acid.

product, $C_{12}H_{18}N_2O$, indicated that a new double bond, conjugated to the pyrazine ring, had been formed. Location of the hydroxyl group at the tertiary position of the *sec*-butyl side chain (VI in Fig. 2) would be consistent with the facile dehydration which was also observed when hydroxyaspergillic acid was heated to 150° in syrupy phosphoric acid solution, a major portion being thereby converted to a crystalline acidic product, $C_{12}H_{18}N_2O_2$. This compound is still a hydroxamic acid, as shown by the ferric chloride color reaction and the formation of a copper salt. Since it contains 2 less hydrogen atoms than aspergillic acid, it is called dehydroaspergillic acid. The presence of the ethylenic bond was demonstrable by the ready reduction of potassium permanganate solution and by the cleavage with ozone. The ultraviolet absorption spectrum was essentially

identical with that of the hydriodic acid reduction product (Fig. 1) which represents a dehydrodeoxyaspergillic acid.

It was hoped that catalytic reduction of the new double bond would yield aspergillic acid and thereby confirm the postulated structure (VI) of hydroxyaspergillic acid by this conversion. However, reduction, even under very mild conditions, did not stop with the absorption of 1 mole of

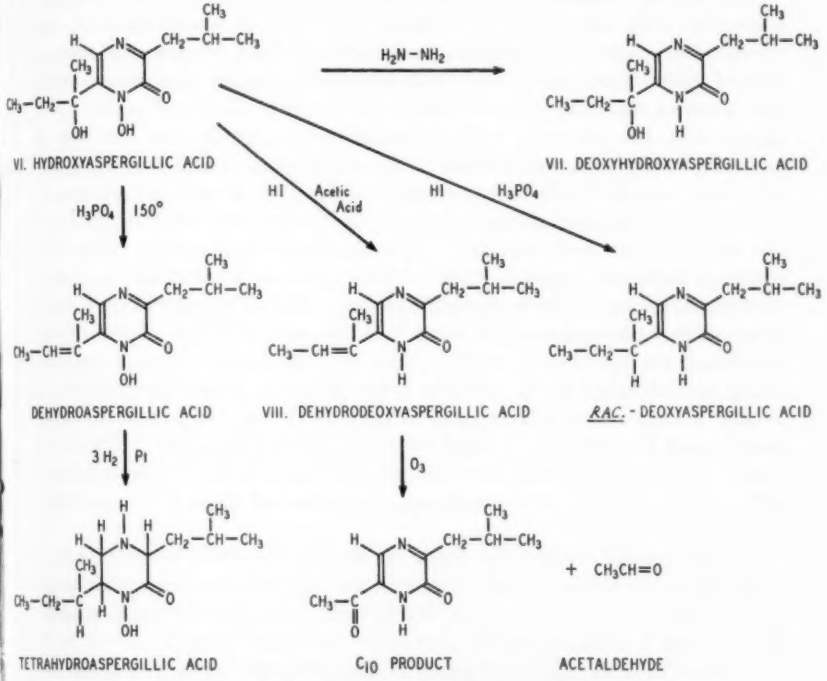


FIG. 2. Products derived from hydroxyaspergillic acid

hydrogen, but, in contradistinction to aspergillic acid which resists catalytic reduction (6), 3 moles of hydrogen were absorbed to yield the compound tetrahydroaspergillic acid, $C_{12}H_{24}N_2O_2$. In this case the heterocyclic ring is reduced but not the hydroxamic acid grouping.

With direct comparison impossible by this route other approaches were explored. The reduction of hydroxyaspergillic acid with hydrazine was examined. This is an alternative method of reduction which in the case of aspergillic acid led to deoxyaspergillic acid (IV) (6). The hydrazine reduction product of hydroxyaspergillic acid proved to be the corresponding deoxyhydroxyaspergillic acid (VII in Fig. 2). The behavior of this prod-

uct on reduction with zinc and acetic acid supplied additional proof that the hydroxyl group is not on the ring. If it were on position 6 in Formula V, reduction with zinc and acetic acid should have yielded the diketopiperazine (III), as was the case with 6-bromodeoxyaspergillic acid (6). The isolated product, however, was not the high melting diketopiperazine, but a low melting product with only 1 oxygen atom.

Yet another product which served to confirm Formula VI for hydroxyaspergillic acid and which, in addition, required the modification of the previous structure for aspergillic acid itself was that obtained by reduction of hydroxyaspergillic acid with hydriodic acid in phosphoric acid. The product resulting from this reduction was not the same as that obtained from the reduction with hydriodic acid in acetic acid but was a neutral product which contained 2 hydrogen atoms more ($C_{12}H_{20}N_2O$). Although isomeric with deoxyaspergillic acid (IV), it was not identical. It had nearly the same melting point, crystal form, and solubility properties, but it was optically inactive. This reaction had apparently taken the course of reductive removal of the tertiary hydroxyl group with concomitant racemization of the asymmetric center. But if aspergillic acid, and presumably hydroxyaspergillic acid, had *two* *sec*-butyl side chains, each containing an asymmetric carbon atom, the loss of one of these centers would have resulted in an optically active product. There can therefore be only *1* asymmetric carbon atom in these products, and one of the side chains must be either an *n*-butyl or an *iso*-butyl group, not a *sec*-butyl group. Examination of all the other products derived from hydroxyaspergillic acid by removal of the hydroxyl group showed them to be optically inactive.

The subsequent reports by Dunn *et al.* and Newbold *et al.* (13–15), which led to the revision of the nature of the side chains in aspergillic acid and established their location on the pyrazine ring, enable the products derived from hydroxyaspergillic acid to be correlated (Fig. 2).

Evidence that the hydroxyl group is located in the tertiary position of the *sec*-butyl side chain was afforded by the following observations: (1) Neither hydroxyaspergillic acid nor deoxyhydroxyaspergillic acid could be acylated under the usual conditions for esterifying a secondary alcohol. (2) On treatment with hypoiodite neither product yielded iodoform, which would have been formed from the grouping CH_3CHOH—. (3) On ozonolysis, dehydrodeoxyaspergillic acid yielded acetaldehyde (characterized as the 2,4-dinitrophenylhydrazone) and a compound, $C_{10}H_{14}N_2O_2$, which was optically inactive and gave a positive iodoform reaction. These products would be those expected from a compound of the structure shown in Formula VIII (Fig. 2).

Thus, with the structure of aspergillic acid (V) clearly established (15)

and the correlation established between hydroxyaspergillic acid and aspergillic acid through the conversion of the former to racemic deoxyaspergillic acid, the evidence presented in the foregoing paragraph for placing the hydroxyl group at the tertiary position of the *sec*-butyl side chain clearly establishes the structure of hydroxyaspergillic acid as that shown in VI.

In view of the observation that various cyclic hydroxamic acids have considerable antibacterial activity (17, 18), it is of interest to compare the activities of the two derived products, dehydroaspergillic acid and tetrahydroaspergillic acid, with those of aspergillic acid and hydroxyaspergillic acid. These are shown in Table II.

TABLE II

Comparison of Activities of Dehydroaspergillic and Tetrahydroaspergillic Acids with Those of Aspergillic and Hydroxyaspergillic Acids

	Minimal inhibiting concentration, γ per ml. vs.				
	Streptococcus pyogenes C_2O_3	*Micrococcus pyogenes* var. *aureus*	*Klebsiella pneumoniae*	BCG	*Escherichia coli*
Aspergillic acid	3	10	30	4	30
Hydroxyaspergillic acid	40	100	150	8	250
Dehydroaspergillic "	4	15	60	2	25
Tetrahydroaspergillic acid	45	100	200	40	400

EXPERIMENTAL

Hydroxyaspergillic Acid—Although purified aspergillic acid can be readily obtained from crude preparations which are mixtures of aspergillic acid and hydroxyaspergillic acid by repeated crystallization from hexane (6), it is much more difficult to obtain the hydroxyaspergillic acid free from aspergillic acid by such procedures. Countercurrent solvent distribution (of the mixed product) between ether and 25 per cent sulfuric acid is able to separate them satisfactorily but is laborious. Sufficient quantities of hydroxyaspergillic acid for structural investigation were obtained by selecting material from those fermentations in which only hydroxyaspergillic acid was produced (5, 16). After several recrystallizations from hexane, the melting point was constant at 149–150°.[1] Pure hydroxyaspergillic acid is only very slightly soluble in ether or hexane at 25° but is readily soluble in chloroform, acetone, methanol, or ethanol. It is highly insoluble in water but dissolves readily in 0.1 N sodium hydroxide solution. By potentio-

[1] All melting points were determined in a capillary tube in an oil bath and are uncorrected.

metric titration a neutralization equivalent of 240 was obtained (pK_a = 4.9).

$C_{12}H_{20}N_2O_3$ (240.3).	Calculated.	C 59.98, H 8.39, N 11.66
	Found.[3]	" 60.01, " 8.25, " 11.74

Copper Salt of Hydroxyaspergillic Acid—An ethanolic solution of the acid was treated with an ethanolic solution of cupric acetate; on addition of water the salt crystallized as grass-green rectangular plates or cubes, m.p. 216°.

$(C_{12}H_{19}N_2O_3)_2Cu$ (542.14).	Calculated.	C 53.10, H 7.01, N 10.32, Cu 11.74
	Found.	" 52.88, " 7.14, " 10.20, " 11.72

Unless excess of cupric acetate was present a crystalline, basic copper salt was obtained, m.p. 215°.

$(C_{12}H_{19}N_2O_3)CuOH$ (319.6).	Calculated.	C 45.10, H 6.26, N 8.76, Cu 19.9
	Found.	" 45.36, " 6.28, " 8.96, " 21.0

Dehydrodeoxyaspergillic Acid—A solution of 5.0 gm. of hydroxyaspergillic acid in 25 ml. of glacial acetic acid was added to a solution containing 1.0 gm. of iodine and 1.0 gm. of red phosphorus in 25 ml. of glacial acetic acid. The mixture was refluxed for 2 hours and then filtered through sintered glass into 200 ml. of a 1 per cent aqueous solution of sodium bisulfite. A pale yellow precipitate formed and was filtered off and washed with water. The yield of dried material was 2.98 gm. This was crystallized from acetone; long, colorless prismatic rods (with m.p. 158°) were obtained.

$C_{12}H_{18}N_2O$ (206.3).	Calculated.	C 69.87, H 8.79, N 13.6
	Found.	" 69.74, " 8.76, " 13.9

A solution in chloroform (c = 2.6) had no optical rotatory activity.

Dehydroaspergillic Acid—The dehydration of hydroxyaspergillic acid was found to proceed smoothly in 85 per cent syrupy phosphoric acid. A solution of 1.09 gm. in 17 ml. was warmed to 150° in an oil bath. The course of the reaction could be followed by determining the ultraviolet spectrum at intervals. In this solvent hydroxyaspergillic acid showed absorption maxima at 245 and 363 mμ with $E^{1\%}_{1\,cm.}$ = 410 and 310, respectively. As the dehydroaspergillic acid was formed a shoulder appeared at 360 mμ and eventually a distinct maximum was obtained at 335 mμ with $E^{1\%}_{1\,cm.}$ = 445. At this point the reaction mixture was cooled, poured into 5 volumes of cold water, and extracted with chloroform. The material in the chloroform layer was separated into neutral and acidic fractions by extraction with 5 per cent sodium carbonate solution. There were ob-

[3] All analyses were carried out by Mr. J. F. Alicino, The Squibb Institute for Medical Research, New Brunswick, New Jersey.

tained 290 mg. of neutral material and 360 mg. of acidic material. The neutral fraction crystallized readily from ethanol as long, colorless prisms, m.p. 158°, and was identical with dehydrodeoxyaspergillic acid as described previously. The acidic substance crystallized directly on acidification of the sodium carbonate solution. Pale yellow prisms were obtained on recrystallization from aqueous ethanol, m.p. 116–117°.

$C_{12}H_{16}N_2O_2$ (222.3).	Calculated.	C 64.83, H 8.16, N 12.60
	Found.	" 64.88, " 8.14, " 12.37

The ultraviolet absorption spectrum in ethanol is shown in Fig. 1. A concentrated solution in ethanol showed no optical activity. A methanolic solution of this acid gives an intense red color with ferric chloride solution and forms a deep green copper salt, m.p. 224–225°.

$(C_{12}H_{17}N_2O_2)_2Cu$ (506.11).	Calculated.	C 56.94, H 6.77, N 11.07, Cu 12.57
	Found.	" 56.99, " 6.96, " 10.82, " 11.74

Ozonolysis of Dehydrodeoxyaspergillic Acid—A solution of 671 mg. of dehydrodeoxyaspergillic acid, m.p. 158°, in 25 ml. of chloroform was ozonized for 30 minutes, during which time an amount of ozone in slight excess of 1 equivalent was passed into the solution. The chloroform was removed *in vacuo*, and the oily residue was steam-distilled after the addition of 50 ml. of water. The distillate was collected in Brady's reagent, and the yellow precipitate which formed was recrystallized from ethyl acetate. The melting point and analysis were in agreement with those of the 2,4-dinitrophenylhydrazone of acetaldehyde.

$C_8H_8N_4O_4$ (224.18).	Calculated.	C 42.85, H 3.60, N 25.00
	Found.	" 43.00, " 3.64, " 24.81

The non-volatile product was extracted with chloroform which on evaporation yielded a crude crystalline residue weighing 568 mg. Recrystallization from acetone gave dense, pale yellow prisms, m.p. 113–114°, after sintering at 109°.

$C_{10}H_{14}N_2O_2$ (194.23).	Calculated.	C 61.84, H 7.26, N 14.43
	Found.	" 61.63, " 7.21, " 14.23

This compound was optically inactive; it gave an immediate precipitate of iodoform on treatment with hypoiodite.

Tetrahydroaspergillic Acid; Catalytic Hydrogenation of Dehydroaspergillic Acid—Reduction of dehydroaspergillic acid in either ethanol or acetic acid with reduced platinum oxide as catalyst led to the rapid absorption of 3 moles of hydrogen. There was no diminution in the rate of absorption at 1 mole of hydrogen. The acidic, syrupy reduction product could not be induced to crystallize; hence, it was converted to the cupric salt which

crystallized from aqueous dioxane as dark green prisms, m.p. 209–210°. Decomposition of the copper salt (6) yielded the acid which was crystallized, but with difficulty, from a small volume of aqueous ethanol, m.p 75–80°. A crystalline picrate was obtained by treatment of an aqueous ethanolic solution with picric acid; after recrystallization from aqueous acetic acid, it melted at 195–196° with decomposition.

$C_{12}H_{24}N_2O_2 \cdot C_6H_3N_3O_7$ (457.43). Calculated. C 47.26, H 5.95, N 15.31
Found. " 47.34, " 6.04, " 15.10

The free acid was optically inactive and gave a deep red color with ferric chloride solution.

Deoxyhydroxyaspergillic Acid—3 gm. of hydroxyaspergillic acid were dissolved in 20 ml. of ethanol, and 5 ml. of 85 per cent hydrazine hydrate were added. The solution was placed in a sealed tube and heated at 180° for 5 hours. The contents of the tube gave no color with ferric chloride solution. After removal of the ethanol *in vacuo*, the syrupy residue was dissolved in water, neutralized with hydrochloric acid, and extracted with ether. The ether residue crystallized readily when moistened with hexane, and the colorless material could be recrystallized from this solvent. The yield was 1.96 gm.; m.p. 104–105°, $[\alpha]_D$ +21.2° (0.6 in ethanol).

$C_{12}H_{20}O_2N_2$ (224.3). Calculated. C 64.25, H 8.98, N 12.49
Found. " 64.37, " 9.04, " 12.37

Racemic Deoxyaspergillic Acid—To a solution of 4.066 gm. of hydroxyaspergillic acid in 40 ml. of syrupy 85 per cent phosphoric acid were added 1.0 ml. of hydriodic acid (sp. gr. 1.7) and 1.0 gm. of red phosphorus. The mixture was heated in an oil bath at 150–160° for 3 hours at which time the solution no longer gave a color with ferric chloride. The hot solution was filtered through sintered glass into 150 ml. of 1 per cent sodium bisulfite solution, and the resulting aqueous solution was extracted with chloroform. Removal of the chloroform *in vacuo* yielded a semicrystalline mass weighing 3.174 gm. Recrystallization from acetone yielded colorless needles, m.p. 98–100°. Further recrystallization from hexane raised the melting point to 103–104° (13).

$C_{12}H_{20}N_2O$ (208.30). Calculated. C 69.20, H 9.62, N 13.45
Found. " 69.40, " 9.66, " 13.45

This product had all the properties of deoxyaspergillic acid except that it was optically inactive.

SUMMARY

The structure of hydroxyaspergillic acid, an antibiotic produced along with or to the exclusion of aspergillic acid (6) by *Aspergillus flavus*, has

been established. It has been shown to have the same structure as aspergillic acid but with an additional hydroxyl group located at the tertiary position of the *sec*-butyl side chain. The antibacterial activity of hydroxyaspergillic acid and that of some of the derived products have been compared with that of aspergillic acid.

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THE COURSE OF HYDROXYLATION OF LYSINE TO FORM HYDROXYLYSINE IN COLLAGEN*

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(Received for publication, January 20, 1958)

Previous experiments in this laboratory (1, 2) have indicated that the hydroxylysine in rat skin collagen is formed from lysine and not to a significant extent from other sources. Collagen from young growing rats that had received uniformly C^{14}-labeled lysine in their food for 3 weeks was found to have both its lysine and hydroxylysine uniformly labeled and with approximately the same specific activity. Free hydroxylysine itself appeared not to be incorporated into collagen, as indicated by failure to find significant activity in collagen hydroxylysine after administration of tritium-labeled hydroxylysine (2).

In the previous experiments with feeding of C^{14}-lysine the equality in C^{14} specific activity between lysine and hydroxylysine isolated from cartilage indicated that, during or after incorporation of the lysine into the collagen, hydroxylation occurred to such an extent that the two incorporated C^{14}-amino acids reached a ratio, hydroxylysine to lysine, equal to the molecular ratio of the two amino acids in the collagen. Otherwise, differing degrees of dilution with the preexisting lysine and hydroxylysine would have produced different specific C^{14} activities in the two amino acids in the protein. The experiments with C^{14}-lysine feeding for 3 weeks did not, however, indicate whether hydroxylation to the final ratio was completed during incorporation or was a time reaction progressing after incorporation.

The present experiments have been done to obtain information on this question. Growing rats have been given each a single intraperitoneal injection of uniformly C^{14}-labeled lysine, and the C^{14} activities of the lysine and hydroxylysine have been determined in the skin collagen of the rats killed 1 hour, 4 hours, and 2 weeks after the injections.

EXPERIMENTAL

In each experiment four rats, of about 4 weeks age and between 55 and 65 gm. weight, were injected with L-lysine uniformly labeled with C^{14} in

* This research was supported by the Atomic Energy Commission and Eli Lilly and Company. It has been partially reported (Quarterly report of the Brookhaven National Laboratory, Washington, D. C., p. 40 (1957).

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the amounts indicated in Table I.[1] The three groups of rats were sacrificed after 1 hour, 4 hours, and 2 weeks, respectively.

The pelts were removed, and the hair was cut off. The pelts were cut

TABLE I

Specific C^{14} Activities of Lysine and Hydroxylysine Incorporated in Rat Skin Collagen after Injection of C^{14}-Labeled Lysine

Time after injection	Lysine injected into 4 rats		C^{14} mμc. per mg. carbon in amino acids of skin collagen*			
		C^{14}		Lysine	Hydroxy-lysine	Ratio, hydroxylysine / lysine
(1)	(2)	(3)	(4)	(5)	(6)	(7)
hrs.	*mg.*	*μc.*				
1	89.0	53.3		1.950 1.939 1.931	1.319 1.321	
Average				1.940	1.320	0.68
S.e. of counts, %				±0.50	±1.39	
4	61.6	64.8		10.48 9.96 9.90 10.16	7.21 7.17	
Average				10.12	7.19	0.71
S.e. of counts, %				±0.33	±0.67	
wks.						
2	58.4	64.0		0.920 0.940 0.933	1.027 1.008 1.009	
Average				0.932	1.015	1.10
S.e. of counts, %				±0.50	±0.84	

* 1 mμc. corresponds to 2220 disintegrations per minute and to about 2000 c.p.m. in the gas counters (3) used for the $C^{14}O_2$.

into small pieces and soaked in chloroform-methanol and urea, as described in the previous paper (1). They were then boiled under reflux for 40 hours with 300 ml. of water. The residue of skin was separated on a Buchner plate, and the solution was filtered through Whatman No. 42 filter paper. An equal volume of 10 per cent trichloroacetic acid was add-

[1] The lysine was purchased from the Schwarz Laboratories, Inc., 230 Washington Street, Mount Vernon, New York.

ed, and the solution was allowed to stand overnight. The precipitate was removed by centrifugation. The supernatant solution was dialyzed against three changes of distilled water. The small amount of precipitate which appeared was removed by centrifugation, and the supernatant solution was concentrated *in vacuo* to 20 to 25 ml. (In the 4 hour experiment trichloroacetic acid was not used; instead, the gelatin was precipitated with acetone, as described in a previous paper (1)). The solution was hydrolyzed by the addition of an equal volume of concentrated HCl and refluxing for 40 hours.

The basic amino acids, lysine, hydroxylysine, arginine, and histidine, were precipitated as the phosphotungstates and then changed to hydrochlorides, according to the procedure of Van Slyke, Hiller, and MacFadyen (4), as described in a previous paper (1). Lysine and hydroxylysine were separated by chromatography on Dowex 50, the procedure being that previously described, except that 0.1 M citrate buffer, pH 5 (see Hamilton and Anderson (5)), was employed in the present work rather than phosphate. Citrate was removed from the solutions of lysine and hydroxylysine by passing the solutions through a column of Dowex 50, 20 cm. long and 2.5 cm. in diameter. The Dowex 50 was in the acid phase. The basic amino acids were held, and the sodium ions were eluted when 250 ml. of 1 N HCl were passed through the column, according to the procedure of Mueller, Bowman, and Herranen (6). The lysine or hydroxylysine was eluted with 6 N HCl, and the HCl was removed *in vacuo*.

Counting Procedure

Because the conclusions with regard to hydroxylation rate of lysine depend upon percentage differences in C^{14} activities, precautions were taken to limit the combined errors of determining the amounts of lysine and hydroxylysine and the disintegration counts of the samples.

The combined desalted solutions of lysine and hydroxylysine were brought to a volume of either 25 or 30 ml., and the concentrations of amino acid were determined in aliquot portions by the manometric nitrous acid method (7), the amounts available providing for results within a maximal error of ± 0.5 per cent for lysine and ± 1.0 per cent with hydroxylysine.

Other aliquots were dried in Van Slyke-Folch (8) combustion tubes and used for combustion and gas counting of the $C^{14}O_2$ by the method of Van Slyke, Steele, and Plazin (3). The counts per minute varied from about 5000 for lysine in the experiment with 4 hour rats to 400 for hydroxylysine with the 2 week-old group, the backgrounds being 65 to 75 c.p.m. The counts of both background and samples were continued for sufficient durations to bring the standard error of the count below ± 0.9 per cent, except in the case of hydroxylysine for the 1 hour group for which it was ± 1.39

per cent. The observed gross count, $N_G \times t_G$, varied from 25,000 to 100,000.

The standard percentage error of the count was calculated as

$$\text{Per cent } E = \pm \frac{100}{N_s} \sqrt{\frac{N_G}{t_G} + \frac{N_B}{t_B}}$$

Per cent E = standard percentage error, N_G = gross count per minute of sample plus background, N_B = background count, N_S = sample count = $N_G - N_B$. t_G and t_B = minutes used for counting gross and background counts, respectively.

The formula is derived from the conventional equation (Hevesy (9), p. 76) as follows:

$$\sigma_S = \sqrt{\sigma_G^2 + \sigma_B^2}$$

where σ = standard error in counts, and subscripts S, G, and B are explained above. σ_G and σ_B are replaced by their values, $\sqrt{N_G/t_G}$ and $\sqrt{N_B/t_B}$, and per cent E is taken as 100 (σ_S/N_S).

Calculation of C^{14} Retained in Lysine and Hydroxylysine of Skin Collagen

From the amount of nitrogen in the skin collagen preparations (Column 3, Table II) and the percentages of the collagen nitrogen found in lysine (4.7 per cent) and hydroxylysine (0.63 per cent) (average of the values in Columns 4 and 5, respectively, Table II) the amounts of lysine and hydroxylysine nitrogen and carbon (C = N × 72/28) were calculated. From the mg. of collagen lysine and hydroxylysine carbon thus found and the specific activity, millimicrocuries per mg. of carbon (Columns 5 and 6, Table I), the millimicrocuries of C^{14} in the lysine and hydroxylysine of the collagen preparations and their relation to the C^{14} in injected lysine (Column 3, Table I) were calculated. The millimicrocuries in the collagen lysine and hydroxylysine are accordingly calculated as:

Millimicrocuries of collagen lysine C
= (mg. of collagen N) × 0.047 × (72/28)
× (millimicrocuries per mg. of lysine C)

Millimicrocuries of collagen hydroxylysine C = (mg. of collagen N)
× 0.0063 × (72/28) × (millimicrocuries per mg. of hydroxylysine C)

The millimicrocurie values thus calculated are divided by 10 times the microcuries injected (Column 3, Table I) to give the percentages of injected lysine-C^{14} found in the lysine and hydroxylysine of the skin collagen preparations (Columns 6, 7, and 8, Table II).

Lysine and Hydroxylysine Contents of Rat Skin Collagen

Data in the literature (10, 11) indicate that hydroxylysine nitrogen usually constitutes somewhat about 1 per cent of the nitrogen of gelatin

prepared from mammalian skin collagen by methods similar to that used in the present paper, by avoiding treatment by alkali or enzymes. The lysine nitrogen found has been about 4.5 per cent of the total nitrogen.

In our previous paper (1) the areas of the hydroxylysine and lysine peaks obtained in chromatographic separation of the two amino acids from the bases precipitated by phosphotungstic acid (Fig. 1 (1)) indicated only about one-ninth as much hydroxylysine as lysine. It was thought that solubility of hydroxylysine phosphotungstate, which is influenced by co-precipitation with arginine and lysine phosphotungstates, might be responsible for the relatively small proportion of hydroxylysine found in the rat collagen. (The solubility of lysine phosphotungstate is so small that loss from the solubility is negligible (12).)

TABLE II

Lysine and Hydroxylysine Contents of Rat Skin Collagen and Percentages of Injected Lysine-C^{14} Retained in Total Skin Collagen

Time after injection	Total* weight of 4 rats	Collagen* N extracted from skins	Percentage of collagen N in		Percentage of injected lysine-C^{14} found in collagen amino acids†		
			Lysine	Hydroxylysine	Lysine	Hydroxylysine	Total
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
hrs.	*gm.*	*mg.*					
1	230	147	4.62	0.59	0.065	0.006	0.071
4	233	214	4.80	0.66	0.404	0.038	0.442
wks.					0.092	0.013	0.105
2	586	521					

* Each of the three groups was 1 month old at the time of injection. Increase in skin collagen (Column 3) was approximately proportioned to increase in body weight in the 2 week group.

† In calculating the values in Columns 6, 7, and 8 the amounts of lysine and hydroxylysine were calculated on the assumption that their nitrogen constituted 4.7 and 0.63 per cent, respectively, of the collagen nitrogen.

Therefore in the analysis of collagen from the present 4 hour rats quantitative estimates of the lysine and hydroxylysine obtained from the phosphotungstate precipitate were made by determining these amino acids in their respective peaks by Moore and Stein's ninhydrin photometric method, with standards prepared not from leucine but from lysine and hydroxylysine for their respective fractions and by checking the results with the nitrous acid manometric method (7). The results indicated 0.49 per cent of the collagen nitrogen in hydroxylysine and 4.62 per cent in lysine, in the bases precipitated by phosphotungstic acid. To find whether the low hydroxylysine value was due to incomplete precipitation as phosphotungstic acid,

the filtrates and washings from the precipitation and recrystallization of the phosphotungstates were freed of phosphotungstic acid by neutralizing with sodium hydroxide and precipitating the phosphotungstate with barium chloride. The solution was then desalted and chromatographed to determine the hydroxylysine, which was found to be 0.086 per cent of the collagen nitrogen. The total hydroxylysine nitrogen was therefore only 0.58 per cent of the collagen nitrogen.

In the analysis of the collagen from the 1 hour rats 0.2 of the hydrolysate was used for chromatographic determination of the hydroxylysine, as described by Hamilton and Anderson (5) without preliminary precipitation by phosphotungstic acid. This determination gave 0.66 per cent of the collagen nitrogen as hydroxylysine and 4.80 per cent as lysine nitrogen.

The lysine content found agrees with that determined by other authors (10, 11) for the lysine from ox hide gelatin prepared and purified by methods somewhat similar to those used for rat skin gelatin in the present paper; but the young rat skin content of hydroxylysine, 0.59 to 0.66 per cent of the total nitrogen, is only about 60 per cent of that reported for other mammalian skin collagens. In ox skin collagen Chibnall (10) reported 1.2 per cent. Estoe (11) reported 1.0 per cent for purified ox skin gelatin and commercial gelatin from pig and whale skins (Estoe's values in gm. of amino acid are recalculated in terms of nitrogen).

DISCUSSION

Stability of Skin Collagen in Young Rat

From the fact that, of the amount of C^{14}-lysine plus hydroxylysine found in the total skin collagen 4 hours after injection, only one-fourth was found 2 weeks later, it appears that in rats, during the age period between 4 and 6 weeks, the skin collagen undergoes a rather rapid turnover. Data from collagen obtained at other intervals after injection of C^{14}-lysine are desirable for a more definite estimate of the turnover rate, but from the above data it appears to be more rapid in rats 1 month old than indicated by the results that Neuberger *et al.* (13, 14) obtained by feeding C^{14}-glycine.

Significance of Changes in Ratio of Hydroxylysine to Lysine Specific C^{14} Activities in Collagen

The ratio, C^{14} activity of hydroxylysine to C^{14} activity of lysine, in the collagen shortly after C^{14} administration indicates the extent to which the molar ratio of hydroxylysine to lysine in the preexisting collagen is approached by the change of C^{14}-lysine to C^{14}-hydroxylysine in the newly formed collagen. The C^{14} specific activity of each of these amino acids in a hydrolysate of the collagen is determined by the extent of each one's

dilution with the corresponding C^{12}-amino acid of the total collagen. If, for example, the ratio of C^{12}-hydroxylysine to C^{12}-lysine in the total (chiefly preexisting) collagen is 1:8, and labeled collagen is added in which one-ninth of the incorporated C^{14}-lysine is hydroxylated to form hydroxylysine, with the resultant 1:8 molar ratio, the C^{14} activities of the lysine and hydroxylysine in the total collagen will be equal, since each of these C^{14}-labeled amino acids is diluted the same number of times with the C^{12} species. The values, 0.68 and 0.71, for the ratio C^{14} activity of hydroxylysine to C^{14} activity of lysine, found 1 and 4 hours, respectively, after injection of C^{14}-lysine indicate that formation of hydroxylysine had gone to 68 and 71 per cent, respectively, of the extent necessary to give the newly formed collagen the same molar hydroxylysine to lysine ratio as that in the preexisting collagen, the term "collagen" being used to indicate the protein or protein mixture obtained by the procedure of preparation used in the present work.

In the rats killed 2 weeks after the C^{14}-lysine injection, the ratio, C^{14} activity of hydroxylysine to C^{14} activity of lysine, had risen to 1.10. This ratio indicates that after 2 weeks the molar ratio of hydroxylysine to lysine in the labeled amino acids increased from 0.70 to 1.10 times the ratio existing in the mass of the protein analyzed as "collagen."[2]

Any hypotheses to explain the results in this and previous papers (1, 2) must accord with the following apparent facts: (1) Hydroxylysine in collagen originates entirely from hydroxylation of lysine. (2) The ratio of the specific C^{14} activity of hydroxylysine to the activity of lysine in the rat skin collagen is about 0.70 either 1 hour or 4 hours after injection and then rises slowly to reach 1.10 in 2 weeks. (3) Turnover of skin collagen in the young rat is rapid enough to remove in 2 weeks three-fourths of the C^{14}-labeled lysine and hydroxylysine present 4 hours after C^{14}-lysine injection (see Column 8 of Table II), presumably with the collagen molecules containing them.

Of the possible hypotheses the following two are outlined: The first is that the increase in the ratio of specific C^{14} activity of hydroxylysine to the activity of lysine in the skin collagen from 0.68 in the 1st hour after injection to 1.10 2 weeks later is due to a single process, *viz.* hydroxylation of lysine, which changes from a quick procedure at the time of incorporation to one that progresses much more slowly. The collagen is accordingly

[2] After this paper was completed a significant contribution by Piez and Likins (15) appeared which confirms the gradual increase in the ratio of C^{14} activity of hydroxylysine to the activity of lysine in skin collagen of young rats after injection of C^{14}-lysine. In collagen taken 1 hour, 6 hours, and 4 days after the injections, Piez and Likins found ratios of 0.72, 0.73, and 0.96, respectively. Their experiments were not prolonged to the 2 week period, at which we observed the ratio 1.10.

a mixture of young collagen molecules, in which the hydroxylysine to lysine ratio is 0.7 of the ratio in the total collagen, and older collagen molecules, in which the ratio is greater than that in the total collagen. As the young labeled molecules mature, hydroxylation slowly continues; thus after 2 weeks the hydroxylysine to lysine ratio in the labeled, now relatively old, collagen molecules is 1.10 times the average ratio in the collagen. A constant content of hydroxylysine and lysine in the total collagen is maintained by the process of synthesis, maturing, and breakdown of collagen molecules.

A feature of this hypothesis that appears somewhat improbable is the change of the hydroxylation reaction from an almost instantaneous one at the moment of protein synthesis to a very slow one continuing in the protein molecule.

The second hypothesis is based on the assumption that the rat skin "collagen" analyzed consists of two proteins or groups of proteins. One protein is a relatively long lived true collagen which, after C^{14}-lysine injection, incorporates C^{14} and at the same time hydroxylates about one-ninth of it; hence, the ratio of C^{14}-labeled hydroxylysine to C^{14}-labeled lysine is the same as the ratio of C^{12}-hydroxylysine to C^{12}-lysine in the mass of true collagen, with the result that the specific C^{14} activities of the two amino acids in the true collagen are at once equal. The other protein is a relatively short lived, metabolically more active "x-protein," which like most other tissue and blood proteins (13, 14) turns over more rapidly than collagen. The x-protein incorporates lysine but does not hydroxylate it and, like non-collagen proteins in general, contains no hydroxylysine. Assume that, of the two proteins, the x-protein, being metabolically the more active, after injection of C^{14}-lysine incorporates it more rapidly than does the true collagen; thus the specific activity of lysine is greater in the x-protein than in the true collagen. As a result of thus increasing the specific activity of the *lysine* in the protein mixture, the ratio of hydroxylysine to lysine C^{14} activity in the mixture falls below the value 1 that it has in the true collagen, hence the 0.68 and 0.71 ratios in the 1 and 4 hour experiments. As the shorter lived x-protein with its C^{14}-lysine is replaced by C^{12}-x-protein, the specific activity of the lysine in the x-protein gradually falls to below the activity of the lysine in the true collagen, and the hydroxylysine-lysine C^{14} activity ratio in the collagen-x-protein mixture rises to above 1. If the amount of lysine in the x-protein were 10 per cent of the lysine in the true collagen, the complete replacement of labeled x-protein by unlabeled protein could cause the observed 1:1.10 hydroxylysine-lysine activity ratio.

This hypothesis accords, as well as that first outlined, with all the known facts. It provides a more satisfactory explanation for the quick

achievement of the value 0.70 for the ratio, C^{14} activity of hydroxylysine to C^{14} activity of lysine, after injection of C^{14}-lysine and the slower subsequent increase of the ratio to above unity. If the method used for preparing skin collagen and turning it into gelatin failed to remove completely other proteins, their presence could account for the observed changes of the activity ratio on both sides of unity.

At present, the second hypothesis is the one that appears the more probable, *i.e.* formation of hydroxylysine from lysine being simultaneous, or nearly simultaneous, with incorporation of both amino acids into collagen. Whether the hydroxylation occurs in the free lysine, in a peptide precursor of collagen, or in the otherwise complete collagen molecule, present data do not indicate.

SUMMARY

Uniformly C^{14}-labeled lysine was injected intraperitoneally into groups of rats 1 month old and at intervals of 1 hour, 4 hours, and 2 weeks, the skin collagen was analyzed for lysine and hydroxylysine, and their C^{14} specific activities were determined with precautions to avoid errors over 1 per cent.

Of the total collagen nitrogen 4.62 to 4.80 per cent was found in lysine and 0.59 to 0.67 in hydroxylysine. The hydroxylysine content of the young rats' skin collagen is about 0.6 that reported in other mammalian skin collagens.

The ratio of the specific activity of hydroxylysine to that of lysine was 0.68, 0.71, and 1.10, after 1 hour, 4 hours, and 2 weeks, respectively. Reasons are discussed for the probability that hydroxylation of lysine occurred at the time of incorporation into the collagen, the deviations of the activity ratio on both sides of unity being attributable to hydroxylysine-free protein contaminants of the collagen.

The amount of C^{14}-lysine plus hydroxylysine in the total collagen of the skins was one-fourth as great 2 weeks after the injections as after 4 hours, indicating turnover of skin collagen in rats of the age used.

The authors wish to acknowledge the technical assistance of Mr. John Plazin and Miss Barbara Faris.

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GLUCOSAMINE METABOLISM

IV. GLUCOSAMINE-6-PHOSPHATE DEAMINASE*

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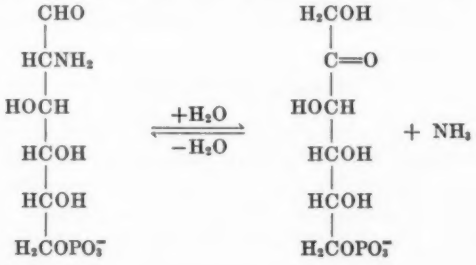
(Received for publication, January 13, 1958)

Early observations on the metabolism of D-glucosamine indicated that this sugar was converted to ammonia and the usual products of glycolysis by bacteria and by mammalian tissue slices (3). Recent studies with extracts obtained from bacteria (4) and from rat brain (5) indicated that glucosamine was transformed to hexose via the phosphate esters. A previous study from this laboratory (6) showed that extracts from *Escherichia coli*, purified to remove phosphohexoisomerase, converted glucosamine 6-phosphate to fructose 6-phosphate and ammonia. The reaction was reversible and no cofactor requirements could be demonstrated. The bacterial enzyme apparently catalyzes the accompanying reaction.



Glucosamine 6-phosphate — Fructose 6-phosphate

The identification of fructose 6-phosphate as the reaction product has also been reported by other laboratories in which extracts from *E. coli* (7) and *Aerobacter cloacae* (8) were used.

With hog kidney extracts, the deamination of glucosamine 6-phosphate

* Preliminary reports have been presented (1, 2). The Rackham Arthritis Research Unit is supported by a grant from the Horace H. Rackham School of Graduate Studies of the University of Michigan. This investigation was supported in part by a grant (No. A-512) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, and a grant from the Michigan Chapter, Arthritis and Rheumatism Foundation.

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was reported (9) to require the addition of *N*-acetylglucosamine 6-phosphate. These extracts also catalyzed the deamination of *N*-acetylglucosamine 6-phosphate *per se*. The following sequence of reactions was suggested

$$N\text{-Acetylglucosamine 6-phosphate} \rightleftharpoons X \quad \text{(I)}$$

$$\text{Glucosamine 6-phosphate} + X \rightleftharpoons N\text{-acetylglucosamine 6-phosphate} + \text{fructose 6-phosphate} + NH_3 \quad \text{(II)}$$

$$X \rightarrow \text{fructose 6-phosphate} + NH_3 + \text{acetate} \quad \text{(III)}$$

Reactions I and II would explain the requirement for *N*-acetylglucosamine 6-phosphate in the deamination of glucosamine 6-phosphate, and Reactions I and III would account for the fact that the extracts acted upon *N*-acetylglucosamine 6-phosphate alone. Further, Compound X was suggested to be *N*-acetylfructosylamine 6-phosphate.

In an attempt to resolve the apparent discrepancy in the reaction mechanisms proposed for the bacterial and hog kidney enzymes, the deaminases from both sources were purified and compared. The results indicate that both enzymes act similarly and that *N*-acetylglucosamine 6-phosphate can activate them but does not participate as an intermediate in the conversion of glucosamine 6-phosphate to fructose 6-phosphate as proposed in Reactions I and II.

Materials and Methods[1]

Materials—Gm-6-P, *N*-Ac-Gm-6-P, Galm-6-P, and *N*-Ac-Galm-6-P were prepared as previously described (10). Isotopically labeled Gm was prepared by growing *Aspergillus parasiticus* in a sucrose-salts medium (11) containing either C^{14}-sucrose or N^{15}-ammonium nitrate. After 8 days growth, the chitin was isolated from the mycelia, was hydrolyzed, and crystalline Gm hydrochloride was isolated after ion exchange chromatography (12). The labeled Gm was phosphorylated with ATP and hexokinase, and the Gm-6-P was isolated and chemically *N*-acetylated (10). Tris, EDTA, TPN (96 per cent purity), protamine sulfate, 2,5-dichlorophenylhydrazine, G-6-P dehydrogenase, and glucosamine hydrochloride were commercial products. Potato phosphatase, free from phosphohexo-

[1] The following abbreviations are used: Gm = glucosamine = 2-amino-2-deoxy-D-glucose; Galm = galactosamine = 2-amino-2-deoxy-D-galactose; *N*-Ac-Gm = *N*-acetylglucosamine = 2-acetamido-2-deoxy-D-glucose; *N*-Ac-Galm = *N*-acetylgalactosamine = 2-acetamido-2-deoxy-D-galactose; Gm-6-P = glucosamine 6-phosphate; Galm-6-P = galactosamine 6-phosphate; *N*-Ac-Gm-6-P = *N*-acetylglucosamine 6-phosphate; *N*-Ac-Galm-6-P = *N*-acetylgalactosamine 6-phosphate; F-6-P = D-fructose 6-phosphate; G-6-P = D-glucose 6-phosphate; Tris = tris(hydroxymethyl)-aminomethane; EDTA = ethylenediaminetetraacetic acid; TPN = triphosphopyridine nucleotide; ATP = adenosine triphosphate; Ac-CoA = acetyl coenzyme A.

isomerase, was obtained by the method of Kornberg.[2] Acetokinase was purified from *E. coli* (13). Gm-6-P *N*-acetylase was prepared from *Neurospora crassa* and purified as described (14).

Analytical Methods—Total hexose was determined by the anthrone procedure (15), F-6-P by the Roe method (16), hexosamine by the method of Blix (17), ammonia by the Conway diffusion procedure (18), *N*-acetylhexosamines by a modified Morgan-Elson method (19), acetate with acetokinase (13), C^{14} with a gas flow counter, and N^{15} with a mass spectrometer.[3] Protein was determined nephelometrically (20) (with crystalline bovine albumin as a standard) during the enzyme fractionations and by micro-Kjeldahl analysis for more accurate determinations.

Enzyme Assay—The reaction mixture for routine estimation of Gm-6-P deaminase activity during purification of *E. coli* extracts contained the following per 0.33 ml.: 5.0 μmoles of Gm-6-P (neutralized immediately before use); 40 μmoles of Tris-HCl buffer, pH 7.8; and 0.1 to 0.2 unit of enzyme. To assay the hog kidney deaminase activity, the reaction mixture was the same except that 1.0 μmole of *N*-Ac-Gm-6-P was included and Tris-HCl buffer at pH 8.9 was used. The reaction was stopped after 15 minutes incubation at 37° by the addition of 0.1 ml. of 2 N HCl. 1 unit of enzyme is defined as the quantity which yields 1.0 μmole of F-6-P in 15 minutes under the above conditions.

Growth of Bacteria—*E. coli* B was grown in a medium containing the following components per liter: 6.0 gm. of Na_2HPO_4; 3.0 gm. of KH_2PO_4; 0.2 gm. of $MgSO_4$; 0.5 gm. of NaCl; and 4.0 gm. of D-glucosamine hydrochloride. The sugar was autoclaved separately (115° for 10 minutes) as a 4 per cent solution and added to the sterilized salt solution. The medium was inoculated with cultures obtained after two serial transplants in this medium. The bacteria were grown for 24 hours with aeration, collected by centrifugation, washed twice with 0.15 M NaCl solution, lyophilized, and stored *in vacuo* at −18°. Extracts of the organism grown on glucose were only one-tenth as active as those prepared from organisms grown on glucosamine (6).

Purification of Enzyme from E. coli

Crude Extract—Unless indicated otherwise, all operations were conducted between 0–4°. The dried cells (10 gm.) were suspended in 100 ml. of 0.02 M potassium phosphate buffer, pH 5.7; 10 ml. aliquots were mixed with 8 gm. of glass beads[4] and the cells were disrupted in a mechanical shaker

[2] Unpublished procedure kindly supplied by Dr. B. L. Horecker.

[3] The N^{15} analyses were performed by Dr. David M. Brown, Department of Chemical and Metallurgical Engineering, University of Michigan.

[4] Superbrite glass beads, No. 115 regular, Minnesota Mining and Manufacturing Company, St. Paul, Minnesota.

(21). The suspension was shaken for 45 seconds, chilled in an ice bath, and again shaken for 45 seconds. The mixture was centrifuged for 1 hour at 21,000 × *g*. and the supernatant fluid dialyzed overnight against 20 volumes of 0.02 M potassium phosphate buffer, pH 5.7. Satisfactory extracts were obtained in early experiments by grinding 3 gm. of dried cells with an equal volume of Alumina (A-301, Aluminum Company of America) and 10 ml. of phosphate buffer, pH 5.7.

Protamine Step—The dialyzed extract (70 ml.) containing 14 mg. of protein per ml. was treated with 20 ml. of a 2 per cent protamine sulfate solution. The mixture was stirred intermittently for 15 minutes, centrifuged, and the precipitate discarded. The clear supernatant fluid should exhibit no turbidity with additional protamine sulfate.

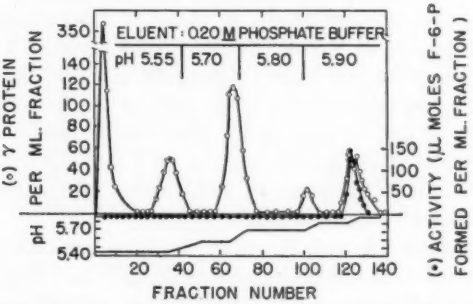


FIG. 1. Chromatography of *E. coli* deaminase on Amberlite XE-64 cation exchange resin.

Chromatography—The solution (64 ml.), containing 1.0 mg. of protein per ml., was added to 6 ml. of Amberlite XE-64 cation exchange resin.[5] The mixture was stirred in an ice bath for 20 minutes, the resin recovered by centrifugation, and the supernatant fluid assayed for enzyme activity. If more than 20 per cent remained in solution, an additional 2 ml. of resin were added and the adsorption was repeated. The resin with adsorbed enzyme was suspended in 0.2 M potassium phosphate buffer, pH 5.40, and transferred to the top of a column (0.9 × 6.0 cm.) of the same resin. Stepwise elution of the protein was accomplished by washing the resin with 0.2 M buffers of increasing pH (Fig. 1). In each case, the column was washed at a flow rate of 1.0 ml. per minute until the pH of the effluent was the

[5] Amberlite XE-64 resin (Rohm and Haas Company) was converted to the acid form by the method of Hirs (22), washed, and suspended in 0.2 M potassium phosphate buffer, pH 5.40. The mixture was adjusted to pH 5.40 with 4 N potassium hydroxide solution and placed on a mechanical shaker. The resin was equilibrated at this pH by the repeated addition of fresh buffer over a period of approximately 16 hours.

same as that of the influent buffer. 5 ml. fractions were collected with an automatic fraction collector. As indicated in Fig. 1, the elution of enzyme coincided with that of the last protein peak. The enzyme purification is summarized in Table I.

The final enzyme preparation was 120-fold purified and displayed no phosphohexoisomerase activity. There was little loss in enzymatic activity over a period of 2 to 3 months at −18° but, after storage for 8 months at −18°, with repeated freezing and thawing, the enzyme lost all activity. Glutathione and EDTA enhanced the activity of the purified enzyme prepared from Alumina-ground cells, but no such effect was noted with enzyme prepared from cells disrupted in the mechanical shaker. There was no indication of a cofactor requirement either during the purification or after

TABLE I

Purification of E. coli Gm-6-P Deaminase

Fraction	Total units*	Specific activity†
Crude extract	17,000	17.6
Protamine step	11,500	185
Amberlite XE-64 resin eluate‡	2,900	2370

* As defined under "Enzyme assay."

† Specific activity was defined as micromoles of F-6-P formed per mg. of protein per 15 minutes at 37°.

‡ Represents total units and average specific activity of Fractions 124 to 127 (Fig. 1).

treatment of the purified enzyme with mixed bed ion exchange resin (Dowex 50, H^+ and Dowex 1, HCO_3^-) or by prolonged dialysis of the purified preparation against EDTA.

Properties of E. Coli Deaminase

Substrate Specificity—Of the substrates tested, the purified enzyme was specific for Gm-6-P. Gm, *N*-Ac-Gm-6-P, Galm-6-P, and *N*-Ac-Galm-6-P were unaffected.

Effect of Substrate Concentration and N-Ac-Gm-6-P—The effect of Gm-6-P concentration on the reaction velocity is illustrated in Fig. 2. The affinity constant (K_m) calculated according to Lineweaver and Burk (23) was 7.1×10^{-3} M. *N*-Ac-Gm-6-P increases the reaction velocity when Gm-6-P is present at levels below that required for saturation of the enzyme (Fig. 3). Under these conditions the calculated K_m was 1.2×10^{-3}, representing a 6-fold decrease in the K_m of the enzyme for Gm-6-P in the presence of *N*-Ac-Gm-6-P. However, the velocity maximum at substrate

saturation is the same in both cases, *i.e.* in the presence or absence of *N*-Ac-Gm-6-P. As will be discussed below, the effect of this compound on the hog kidney deaminase was even more striking.

Effect of pH, Enzyme Concentration, and Incubation Time—The reaction velocity as a function of pH and enzyme concentration is indicated in Figs. 4 and 5. The reaction proceeds at maximal velocity at pH 7.8. Product formation was linear during the time of incubation (Fig. 6).

Characterization of Reaction Product—The product of deamination was

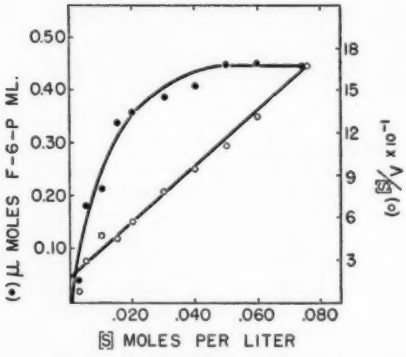


FIG. 2

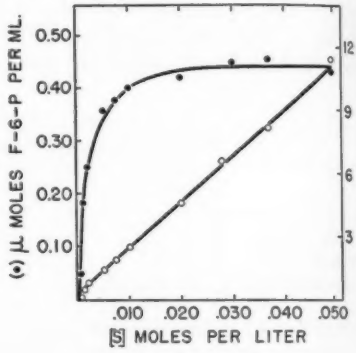


FIG. 3

FIG. 2. Effect of Gm-6-P concentration on the *E. coli* deaminase reaction velocity. In addition to substrate, the reaction mixtures (1.0 ml.) contained 100 μmoles of Tris-HCl buffer, pH 7.8, and 0.2 γ of protein. The incubation time was 15 minutes at 37°.

FIG. 3. Effect of Gm-6-P concentration on the *E. coli* deaminase reaction velocity in the presence of *N*-Ac-Gm-6-P. The reaction mixtures were the same as indicated in Fig. 2, except that *N*-Ac-Gm-6-P at one-tenth the substrate concentration was included.

characterized as F-6-P by the following techniques. Gradient chromatography of the incubated reaction mixture on a Dowex 1 formate column yielded a single peak which coincided with that obtained with authentic F-6-P. Incubation of the product with phosphohexoisomerase and G-6-P dehydrogenase yielded the expected reduction of TPN. After dephosphorylation with potato phosphatase, the neutral sugar was isolated in 70 per cent yield as the 2,5-dichlorophenylhydrazone, m.p. 151°, not depressed by admixture with an authentic sample (24). By paper chromatography (*n*-butanol-ethanol-H_2O, 9:1:10), the dephosphorylated product showed the same R_F as D-fructose (25).

Stoichiometry of Reaction—As indicated in Table II, 1 μmole of Gm-6-P is converted to 1 μmole of F-6-P and 1 μmole of NH_3.

Reversibility Studies—The reaction proceeds largely in the direction of F-6-P and NH_3 formation, but its reversal was detectable under the condi-

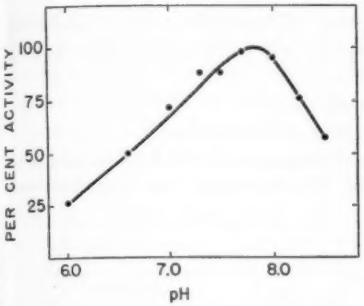


FIG. 4

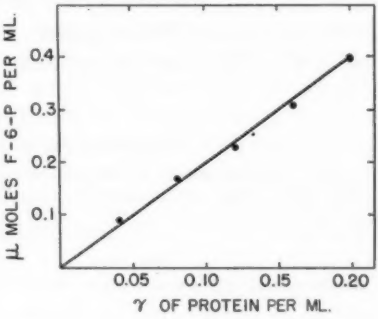


FIG. 5

FIG. 4. Effect of pH on *E. coli* deaminase reaction. The reaction mixtures (1.0 ml.) contained 40 μmoles of Gm-6-P, 120 μmoles of Tris-maleate buffer, pH as indicated, and 0.2 γ of protein. The incubation time was 15 minutes at 37°.

FIG. 5. Effect of *E. coli* deaminase concentration on reaction velocity. The reaction mixtures (1.0 ml.) contained 40 μmoles of Gm-6-P, 100 μmoles of Tris-HCl buffer, pH 7.8, and the indicated amount of protein. The incubation time was 15 minutes at 37°.

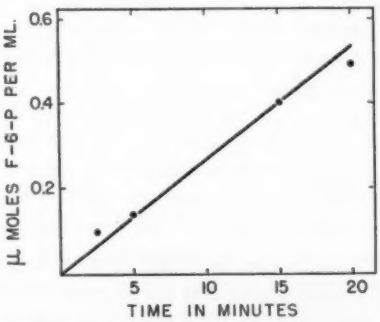


FIG. 6. Effect of incubation time on *E. coli* deaminase reaction. The reaction mixture was the same as indicated in Fig. 5 and contained 0.2 γ of protein. Incubation temperature, 37°.

tions indicated in Table III. *N*-Ac-Gm-6-P did not stimulate the reaction and glutamine did not substitute for NH_3 as the amine donor. The latter fact distinguishes this enzyme from the glutamine-F-6-P transamidase of

N. crassa (26), which catalyzes the synthesis of Gm-6-P from F-6-P and glutamine in an apparently irreversible manner.[6]

Purification of Deaminase from Hog Kidney

Crude Extract—Unless stated otherwise, all operations were conducted at 0–4°. The crude extract was prepared by suspending 1000 gm. of minced fresh hog kidney cortex in 1 liter of 0.02 M potassium phosphate buffer, pH 6.0, and homogenizing 200 ml. aliquots in a Waring blendor for 2 minutes. After centrifugation for 1 hour at 21,000 × *g*, the supernatant fluid was strained through cheesecloth to remove fat and then dialyzed overnight against 20 volumes of 0.02 M potassium phosphate buffer, pH 6.0.

Heat Step—The dialyzed extract was immersed in a water bath at 85°;

TABLE II

Stoichiometry of E. coli Deaminase Reaction

The reaction mixture (1.0 ml.) contained 5.0 μmoles of Gm-6-P, 85 μmoles of Tris-HCl buffer, pH 7.8, and 5 γ of protein. The incubation time was 15 minutes at 37°.

Compound	
	μmoles per ml.
Gm-6-P	−2.38
F-6-P	+2.35
NH_3	+2.32

TABLE III

Reversibility of E. coli Deaminase

Each reaction mixture (1.0 ml.) contained 10 μmoles of F-6-P, 100 μmoles of Tris-HCl buffer, pH 7.8, and 4.5 γ of protein. Additions to this reaction mixture were at 10 μmoles per ml. The incubation time was 30 minutes at 37°.

Additions	Gm-6-P formed
	μmole per ml.
NH_4Cl	0.31
" + *N*-Ac-Gm-6-P	0.22
None	0.00
Glutamine	0.00
" + *N*-Ac-Gm-6-P	0.00

Heat Step—The dialyzed extract was immersed in a water bath at 85°;

[6] The transamidase reaction was not measurably reversible when a partially purified *N. crassa* extract was incubated with Gm-6-P and glutamic acid alone, or in the presence of phosphohexoisomerase, G-6-P dehydrogenase, and TPN. These experiments were performed by Dr. Harold J. Blumenthal.

when its temperature reached 65°, it was immediately transferred to a water bath maintained at 65°, held at this temperature for 2 minutes, cooled rapidly, and centrifuged. The precipitate was discarded.

Ammonium Sulfate I—The supernatant fluid from the heat step was brought to 35 per cent saturation with solid ammonium sulfate and the mixture was mechanically stirred for 30 minutes. After centrifugation, the precipitate was discarded, and the supernatant fluid was brought to 50 per cent saturation with solid ammonium sulfate. After 30 minutes with mechanical stirring, the precipitate was collected by centrifugation, dissolved in 200 ml. of 0.02 M potassium phosphate buffer, pH 5.7, and dialyzed with stirring for 4 hours against 20 volumes of the same buffer. Occasionally, an inactive precipitate formed during dialysis and was removed by centrifugation.

Calcium Phosphate Gel Step—The dialyzed ammonium sulfate fraction, containing 1.05 gm. of protein per 190 ml., was treated with 91 ml. of calcium phosphate gel (32 mg. of solid per ml. (27)). After 20 minutes of gentle stirring, the gel was recovered by centrifugation and the protein eluted by stirring for 20 minutes (each time) with the following 0.05 M potassium phosphate buffers in the indicated order: 200 ml., pH 6.0 (Eluate I); 200 ml., pH 6.5 (Eluate II); 100 ml., pH 7.0 (Eluate III); 100 ml., pH 7.3 (Eluate IV). About 50 per cent of the total enzyme units of the preceding step was present in Eluates III and IV.

Ammonium Sulfate II—Eluates III and IV were combined and adjusted to 50 per cent saturation with solid ammonium sulfate. The precipitate was collected by centrifugation, dissolved in 10 ml. of 0.02 M potassium phosphate buffer, pH 5.7, and dialyzed with stirring for 4 hours against the same buffer. Occasionally, an inactive precipitate formed during dialysis and was removed by centrifugation.

Acetone Step—The dialyzed ammonium sulfate fraction (10 ml.) was adjusted to pH 6.20 with approximately 0.1 ml. of 4 N acetate buffer, pH 5.5. If the pH of the solution dropped below 6.0 in the presence of acetate, the enzyme precipitated and was not readily redissolved. The solution was placed in a bath maintained between −3° and −5°, and acetone was slowly added with stirring until the acetone concentration was 18 per cent (volume per volume). After 5 minutes the precipitate was collected by centrifugation at −5° and suspended in 5 ml. of 0.05 M potassium phosphophosphate buffer, pH 7.0. After 30 minutes with occasional stirring, the mixture was centrifuged and the undissolved solid discarded.

Precipitation at pH 5.70—The acetone fraction was immediately dialyzed against 2 liters of 0.05 M acetate buffer, pH 5.70, for 24 hours, with mechanical stirring. The precipitate was collected by centrifugation and suspended in 5.0 ml. of 0.05 M potassium phosphate buffer, pH 6.50, and stirred

occasionally for 30 minutes. The solid which had not dissolved after this period of time was removed by centrifugation and discarded.

As indicated in Table IV, the enzyme at this point, designated HK-I, is approximately 450-fold purified. When subjected to starch gel electrophoresis (28),[7] two major components and a possible minor component were detected. Repeated precipitation of HK-I at pH 5.70 in acetate buffer yielded an enzyme preparation purified 1360-fold. This preparation, as well as HK-I, catalyzed the conversion of both *N*-Ac-Gm-6-P and Gm-6-P to F-6-P and NH_3. However, when HK-I was repeatedly frozen and thawed, for routine use, over a period of about 1 month, it lost all activity

TABLE IV

Purification of Hog Kidney Deaminase

Fraction	Total units*	Specific activity†
Crude extract	9700	0.57
Heat step	9700	2.8
Ammonium sulfate I	8300	8.6
Calcium phosphate gel (Eluates III + IV)	3900	33
Ammonium sulfate II	3700	58
Acetone, 0–18%	2500	190
Pptn. (HK-I), pH 5.70	1650	260
Repptn. (twice), pH 5.70	870	780

* As defined under "Enzyme assay."

† Specific activity was defined as micromoles of F-6-P formed per mg. of protein per 15 minutes at 37°. The protein analyses, either by the nephelometric or micro-Kjeldahl procedure, indicated the same over-all purification.

against *N*-Ac-Gm-6-P and catalyzed only the conversion of Gm-6-P to F-6-P and NH_3. Henceforth, this preparation will be designated as HK-II.

Phosphohexoisomerase was removed during the fractionation procedure. This separation could also be accomplished at any stage of the purification procedure by precipitation of the protein at 50 per cent ammonium sulfate saturation, dissolving the precipitate in a minimal volume of 0.05 M potassium phosphate buffer, pH 6.0, and heating the solution at 70° for 5 minutes. Approximately 80 per cent of the deaminase activity remained in solution while the isomerase activity was destroyed by the heat treatment.

Properties of Hog Kidney Deaminase

Substrate Specificity—The purified enzyme preparation, HK-I, catalyzed the deamination of either Gm-6-P or *N*-Ac-Gm-6-P. After freezing and

[7] The starch gel electrophoresis experiments were performed through the courtesy of Dr. Walter Block and Dr. Frederick H. Epstein, Department of Dermatology, University of Michigan.

thawing (HK-II), the enzyme preparation catalyzed only the deamination of Gm-6-P, and was completely inactive with *N*-Ac-Gm-6-P (Table V). The following substrates were not deaminated: Galm-6-P; *N*-Ac-Galm-6-P; Galm-6-P plus *N*-Ac-Gm-6-P; Galm-6-P plus *N*-Ac-Galm-6-P; Gm. For purposes of comparison, the substrate specificity of the *E. coli* deaminase is also included in Table V.

Effect of N-Ac-Gm-6-P—As illustrated in Fig. 7, the concentration of activator (*N*-Ac-Gm-6-P) greatly affected the rate of reaction. These results are similar to those reported by Leloir and Cardini (9). At substrate saturation, the maximal velocity of the reaction with HK-II was increased 5-fold in the presence of *N*-Ac-Gm-6-P (Table V). Maximal activation occurred when the ratio of *N*-Ac-Gm-6-P to Gm-6-P was 1:10.

TABLE V

Substrate Specificity of E. coli and Hog Kidney Deaminases

Substrate	Activator	Relative specific activity*	
		Hog kidney (HK-II)	*E. coli*
Gm-6-P	*N*-Ac-Gm-6-P	20	100
"	*N*-Ac-Galm-6-P	10	
"	None	4	100
N-Ac-Gm-6-P		0†	0

* The specific activity is expressed as the per cent activity obtained with *E. coli* deaminase plus Gm-6-P at optimal conditions for each mixture.

† Prior to freezing and thawing (HK-I), the relative specific activity was 13.

The deamination of Gm-6-P by the hog kidney enzyme was found to be activated by *N*-Ac-Galm-6-P as well, although higher concentrations were required for optimal effect (Fig. 7).

Effect of pH—The reaction velocity with HK-I as a function of pH is illustrated in Fig. 8. Gm-6-P alone (not illustrated) yielded a broad maximum between pH 7.0 and 8.2. Gm-6-P plus *N*-Ac-Gm-6-P or *N*-Ac-Galm-6-P gave well defined peaks at pH 8.8 and 7.5, respectively. *N*-Ac-Gm-6-P alone yielded a double peak which suggested the possibility of two enzymes acting on this substrate.

Effect of Enzyme Concentration and Time—The reaction rate was linear with protein concentration with Gm-6-P alone or with either activator (Fig. 9). Under these conditions, the reaction was linear with time for at least 15 minutes.

Effect of Substrate Concentration—The reaction velocity of HK-II as a function of substrate concentration, with and without activator, is indicated in Figs. 10 and 11. The calculated K_m with Gm-6-P alone was 1.4 ×

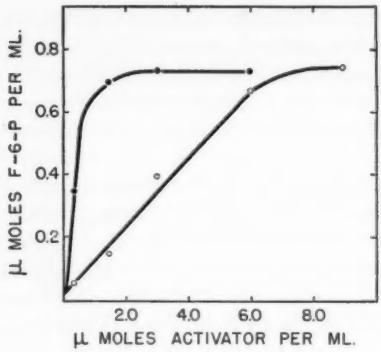


FIG. 7

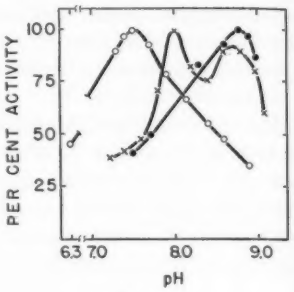


FIG. 8

FIG. 7. Effect of activator concentration on the hog kidney deaminase reaction velocity. The reaction mixtures (1.0 ml.) contained the following: ●, 15 μmoles of Gm-6-P, *N*-Ac-Gm-6-P as indicated, 120 μmoles of Tris-HCl buffer, pH 8.9, and 3 γ of protein (HK-I) specific activity 360; ○, 15 μmoles of Gm-6-P, *N*-Ac-Galm-6-P as indicated, 120 μmoles of Tris-HCl buffer, pH 7.5, and 6 γ of HK-I. The incubation time was 15 minutes at 37°.

FIG. 8. Effect of pH on reaction velocity. The reaction mixtures (1.0 ml.) contained the following: ●, 15 μmoles of Gm-6-P, 3 μmoles of *N*-Ac-Gm-6-P, 120 μmoles of Tris-HCl buffer, and 3 γ of HK-I; ○, 15 μmoles of Gm-6-P, 7.5 μmoles of *N*-Ac-Galm-6-P, 120 μmoles of Tris-HCl buffer (Tris-maleate at pH 6.3), and 12 γ of HK-I; ×, 15 μmoles of *N*-Ac-Gm-6-P, 120 μmoles of Tris-HCl buffer, and 3 γ of HK-I. Under the latter conditions at pH 8.0, 0.44 μmole of F-6-P was formed. The incubation time was 15 minutes at 37°.

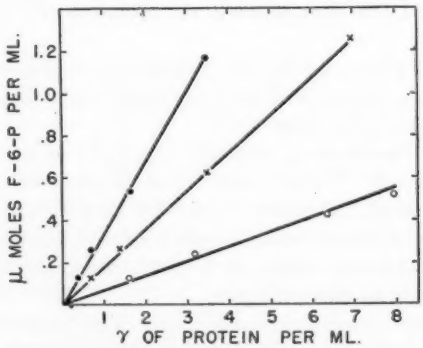


FIG. 9. Effect of hog kidney enzyme concentration on reaction velocity. The reaction mixtures (1.0 ml.) contained the following: ●, 10 μmoles of Gm-6-P, 3 μmoles of *N*-Ac-Gm-6-P, 120 μmoles of Tris-HCl buffer, pH 8.9, and HK-I as indicated; ×, 10 μmoles of Gm-6-P, 9 μmoles of *N*-Ac-Galm-6-P, 120 μmoles of Tris-HCl buffer, pH 7.5, and HK-I; ○, 40 μmoles of Gm-6-P, 100 μmoles of Tris-HCl buffer, pH 7.8, and enzyme preparation HK-II. The incubation time was 15 minutes at 37°.

10^{-2} M. In the presence of *N*-Ac-Gm-6-P, however, the K_m was 6.6×10^{-4} M. It is of interest that HK-II and the *E. coli* deaminase exhibit about the same K_m value with Gm-6-P alone.

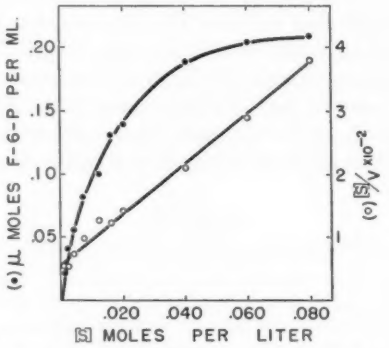


FIG. 10. Effect of Gm-6-P concentration on the hog kidney deaminase reaction velocity in the absence of activator. In addition to substrate, the reaction mixtures (1.0 ml.) contained 100 μmoles of Tris-HCl buffer, pH 7.8, and 3.8 γ of HK-II. The incubation time was 15 minutes at 37°.

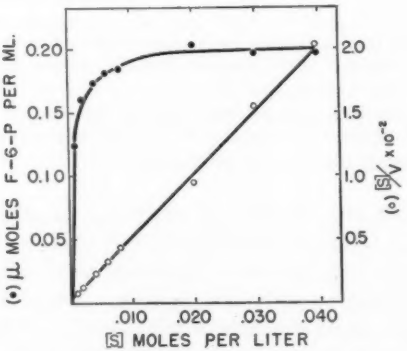


FIG. 11. Effect of Gm-6-P concentration on the hog kidney deaminase reaction velocity in the presence of activator. In addition to substrate, the reaction mixtures (1.0 ml.) contained *N*-Ac-Gm-6-P at one-tenth the Gm-6-P concentration, 100 μmoles of Tris-HCl buffer, pH 8.9, and 0.76 γ of HK-II. The incubation time was 15 minutes at 37°.

Characterization of Reaction Product—The product of the reaction in which Gm-6-P and *N*-Ac-Gm-6-P were employed was characterized as F-6-P in a similar manner to that described for the *E. coli* enzyme. In ad-

dition, the product obtained when *N*-Ac-Galm-6-P was employed as activator exhibited the same R_F as F-6-P by paper chromatography (methanol-formic acid-H_2O, 16:3:1 (29)).

Stoichiometry—The stoichiometric conversion of Gm-6-P to F-6-P and NH_3 by HK-II is indicated in Table VI. Acetate was not detected (acetokinase assay) and no *N*-Ac-Gm-6-P or *N*-Ac-Galm-6-P disappeared.

Reversibility Studies—When HK-II was incubated with F-6-P and NH_4Cl, a small amount of Gm-6-P was formed (Table VII). In the presence of *N*-Ac-Gm-6-P, however, there was a considerable increase in the Gm-6-P formed. The effect of *N*-Ac-Gm-6-P in the deamination reaction

TABLE VI

Stoichiometry with Hog Kidney Enzyme

	Gm-6-P	
	N-Ac-Gm-6-P*	*N*-Ac-Galm-6-P†
	μmoles	*μmoles*
Gm-6-P	−2.03	−1.66
F-6-P	+2.20	+1.78
NH_3	+2.25	+1.54
Acetate	0.00	0.00
N-Acetylhexosamines	0.00	0.00

* The reaction mixture contained the following per ml.: 5.0 μmoles of Gm-6-P; 0.25 μmole of *N*-Ac-Gm-6-P; 100 μmoles of Tris-HCl buffer, pH 8.9; and 7.6 γ of HK-II. Incubated for 15 minutes at 37°.

† The reaction mixture contained the following per ml.: 5.0 μmoles of Gm-6-P; 2.5 μmoles of *N*-Ac-Galm-6-P; 100 μmoles of Tris-HCl buffer, pH 7.4; and 15.2 γ of HK-II. The incubation time was 15 minutes at 37°.

(conversion of Gm-6-P to F-6-P and NH_3) was noted above, and it was demonstrated that maximal activation of the enzyme by *N*-Ac-Gm-6-P occurred when the incubations were conducted at substrate concentration less than that required for enzyme saturation. Since the optimal conditions for the reverse reaction (F-6-P + NH_3 → Gm-6-P) have not yet been defined, the marked activating effect of *N*-Ac-Gm-6-P indicated in Table VII may result from the specific conditions used in these studies.

Role of N-Ac-Gm-6-P in Reaction

The kinetic studies presented above with both the *E. coli* and hog kidney deaminases suggest that *N*-Ac-Gm-6-P functions as an activator in both reactions, although its effect was more pronounced in the case of the latter enzyme. No indication was obtained that this compound was a necessary

intermediate in either reaction as proposed by Leloir and Cardini (9). More definitive evidence that *N*-Ac-Gm-6-P does not participate in the reaction mechanism of either enzyme was obtained from isotope experiments. If Gm-6-P is converted to F-6-P through the intermediate formation of *N*-Ac-Gm-6-P (*e.g.* Reactions I, II, III), labeled *N*-Ac-Gm-6-P should give rise to labeled F-6-P early in the reaction. Since the total quantity of *N*-Ac-Gm-6-P remains constant throughout the course of the reaction, the specific activity of the labeled product would be expected to decline with time. On the other hand, if *N*-Ac-Gm-6-P activates the enzyme without acting as an obligatory intermediate in the reaction, as suggested by the kinetic data, then the products should not be labeled at any stage of the reaction. To demonstrate this with the *E. coli* enzyme, conditions were selected under which the reaction velocity was considerably greater in the presence of *N*-Ac-Gm-6-P than in its absence. The *E. coli* enzyme, in the absence of activator, was found to be sensitive to the type and concentration of buffer employed in the reaction mixture. Thus, in 0.3 M pyrophosphate buffer the reaction rate was considerably decreased unless *N*-Ac-Gm-6-P was present (Fig. 12). An incubation mixture was therefore prepared which contained the following in 1.0 ml.: 11 μmoles of N^{15}-Ac-Gm-6-P (10 per cent atom excess N^{15}); 87 μmoles of Gm-6-P; 300 μmoles of pyrophosphate buffer, pH 7.8; and 10 γ of the purified *E. coli* enzyme. After incubation at 37° for 1 hour, 31 μmoles of NH_3 were produced and were collected in a Conway diffusion apparatus. Assay of the NH_3 indicated no significant amount of excess N^{15}. Based on the errors

TABLE VII

Reversibility Studies with Hog Kidney Enzyme

The complete reaction mixture contained the following per ml.: 50 μmoles of F-6-P; 50 μmoles of NH_4Cl; 150 μmoles of Tris-HCl buffer, pH 8.1; 0.12 μmole of *N*-Ac-Gm-6-P; 9.5 γ of HK-II. The samples were incubated for 1 hour at 37°. F-6-P and NH_4Cl yield high blank values by the acetylacetone method for Gm determination; the samples were therefore analyzed by the acetic anhydride method combined with the modified Morgan-Elson method (30, 19). All samples were corrected for the small blank values obtained with F-6-P, NH_4Cl, and Tris buffer, and for the *N*-Ac-Gm-6-P, where it was added to the incubation mixture.

Incubation mixture	Gm-6-P formed
	μmoles per ml.
Complete	2.15
Omit *N*-Ac-Gm-6-P	0.18
" HK-II	0.00
" F-6-P	0.00

of the method, less than 0.07 per cent of the NH_3 was estimated to be derived from the *N*-Ac-Gm-6-P.

The parallel experiment with the hog kidney deaminase was conducted with C^{14}-*N*-Ac-Gm-6-P labeled in the glucosamine chain. An incubation mixture was prepared containing the following in 10 ml.: 20 μmoles of C^{14}-*N*-Ac-Gm-6-P (1200 c.p.m. per μmole); 200 μmoles of Gm-6-P; 2000 μmoles of Tris-HCl buffer, pH 8.9; and 300 γ of HK-II. The mixture was incubated at 37° and the following aliquots were withdrawn at the indicated times: 5.0 ml. at 2.0 minutes, 2.5 ml. at 5.0 minutes, 1.0 ml. at 10 minutes, and 1.0 ml. at 30 minutes. The samples were heated for 1 minute at 100° and applied to the top of Dowex 1 formate resin (200 to 400 mesh) columns

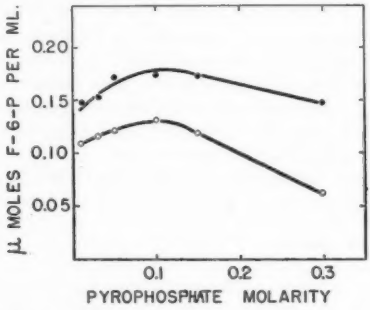


FIG. 12. Effect of pyrophosphate concentration on *E. coli* deaminase. The reaction mixtures (1.0 ml.) for the top curve (●) contained 15 μmoles of Gm-6-P, 3 μmoles of *N*-Ac-Gm-6-P, pyrophosphate buffer, pH 7.8, as indicated, and 0.38 γ of protein. The reaction mixtures for the bottom curve (1 ml.) were the same except that *N*-Ac-Gm-6-P was omitted. The incubation time was 15 minutes at 37°.

(0.9 × 25 cm.). The columns were eluted in a gradient manner with formic acid (750 ml. of 0.05 N formic acid in the mixing vessel and 2.0 N formic acid in the reservoir) and 5 ml. fractions were collected at a flow rate of 0.75 ml. per minute. Gm-6-P appears in the first few fractions. The separation of *N*-Ac-Gm-6-P and F-6-P in the 2.0 minute sample is illustrated in Fig. 13, together with the *total* radioactivity of each fraction. Fig. 13 shows that no radioactive peak corresponding to the F-6-P peak was detectable. The separation of the two sugars and the distribution of radioactivity in the fractions obtained from the 5, 10, and 30 minute samples were identical with that illustrated for the 2 minute sample. Furthermore, when an artificial mixture of F-6-P and labeled *N*-Ac-Gm-6-P was chromatographed in a similar manner, identical results were obtained.

Enzymatic Synthesis of N-Ac-Gm-6-P—Although the equilibrium for the

deaminase reaction strongly favors the formation of F-6-P and NH_3, the reverse reaction might represent an *in vivo* pathway for the synthesis of Gm-6-P and its derivatives. Although Gm-6-P synthesis is barely detectable when measured directly (Tables III and VII), the equilibrium might be shifted towards Gm-6-P formation by the continuous conversion of Gm-6-P to *N*-Ac-Gm-6-P. As indicated in Fig. 14, *N*-Ac-Gm-6-P is rap-

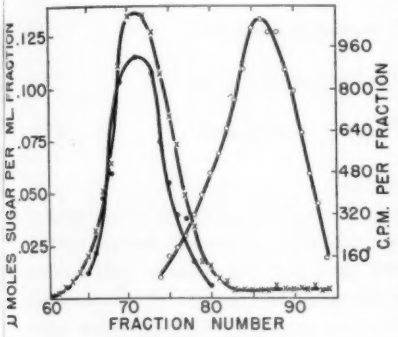


FIG. 13

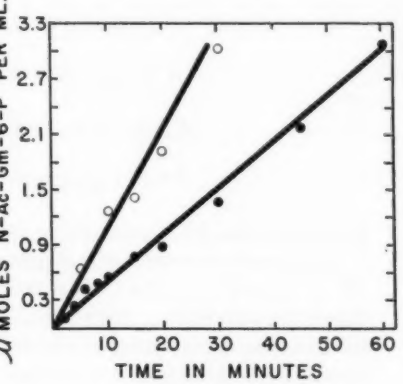


FIG. 14

FIG. 13. Chromatography of 2 minute sample from hog kidney deaminase (HK-II) incubation mixture. Conditions are described in the text. ●, *N*-Ac-Gm-6-P; ○, F-6-P; and ×, counts per minute.

FIG. 14. Enzymatic formation of *N*-Ac-Gm-6-P. The reaction mixtures contained the following per ml.: 33 μmoles of F-6-P; 33 μmoles of NH_4Cl; 17 μmoles of Ac-CoA; 150 μmoles of Tris buffer, pH 8.1; 140 γ of *N. crassa* Gm-6-P acetylase (specific activity, 175 μmoles of product per mg. of protein per 15 minutes). ○, contained 3.3 γ of *E. coli* deaminase; (●) contained 3.3 γ of HK-II. There was no detectable *N*-Ac-Gm-6-P formed when either the deaminase or Gm-6-P acetylase was omitted from the incubation mixture. The addition of 0.15 μmole of *N*-Ac-Gm-6-P to (●) the incubation mixture yielded no increase in *N*-Ac-Gm-6-P formation at any time. The incubation temperature was 37°.

idly formed when either *E. coli* or hog kidney deaminase (used in limiting concentration) was added to a mixture containing F-6-P, NH_3, Ac-CoA, and purified Gm-6-P acetylase (14). The rate of *N*-Ac-Gm-6-P formation was linear with time and in the case of either enzyme was independent of the initial presence or absence of small amounts of *N*-Ac-Gm-6-P. The results obtained with the hog kidney enzyme were unexpected in view of the marked stimulation of *N*-Ac-Gm-6-P on this enzyme either in the forward or the reverse direction. As optimal conditions for Gm-6-P synthesis have not yet been established, the concentration of *N*-Ac-Gm-6-P necessary

for maximal activation of the hog kidney enzyme may not be the same in both directions.

DISCUSSION

The results presented above will be discussed in connection with the following enzymatic reactions

$$\text{Gm-6-P} + \text{H}_2\text{O} \rightleftharpoons \text{F-6-P} + \text{NH}_3 \quad \text{(IV)}$$

$$N\text{-Ac-Gm-6-P} + \text{H}_2\text{O} \rightarrow \text{Gm-6-P} + \text{acetate} \quad \text{(V)}$$

$$\text{Gm-6-P} + \text{Ac-CoA} \rightarrow N\text{-Ac-Gm-6-P} + \text{CoA} \quad \text{(VI)}$$

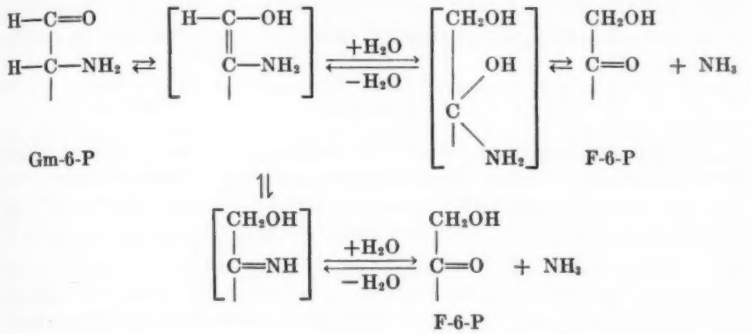
$$\text{F-6-P} + \text{glutamine} \rightarrow \text{Gm-6-P} + \text{glutamate} \quad \text{(VII)}$$

Only Reaction IV is measurably reversible.

In view of our results, the mechanism previously postulated for Reaction IV is no longer tenable. This becomes apparent when the following evidence is considered. Both the *E. coli* and the hog kidney deaminases catalyze the deamination of Gm-6-P without added *N*-Ac-Gm-6-P, and all attempts to dissociate a cofactor from the purified enzymes have been unsuccessful. The kinetic data obtained with the purified enzymes suggest that *N*-Ac-Gm-6-P activates both enzymes similarly with respect to the K_m values, although the maximal velocity remains the same with the *E. coli* enzyme but is increased 5-fold with the hog kidney deaminase. While the mechanism of activation cannot now be explained in molecular terms, the usual derivation of K_m values suggests that *N*-Ac-Gm-6-P (or *N*-Ac-Galm-6-P) increases either the affinity of the enzyme for Gm-6-P or the dissociation of the enzyme-product complex, or that it affects both processes. The isotope data support the evidence for the direct conversion of Gm-6-P to F-6-P and NH_3 and are incompatible with a mechanism which involves the conversion of Gm-6-P to F-6-P and NH_3 through the intermediate formation of *N*-Ac-Gm-6-P. Finally, *N*-Ac-Galm-6-P *per se* apparently activates the hog kidney enzyme. As little as 5 per cent conversion to *N*-Ac-Gm-6-P, formed by transfer of acetyl group to the substrate, would have been detected in the stoichiometry studies since the two *N*-acetylated compounds yield considerable differences in color intensity in the Morgan-Elson method (19).

A situation somewhat similar to the deaminase activation occurs in the conversion of ornithine to citrulline by bacterial and mammalian enzymes (31). In this case, the mammalian enzyme requires the presence of *N*-acyl derivatives of glutamic acid, whereas the bacterial enzyme does not.

The mechanism for Reaction IV is believed to be similar to either of the following



This scheme corresponds to that proposed for the reaction catalyzed by phosphohexoisomerase (32) and for the chemical conversion of fructose and ammonia to glucosamine (33).

Crude preparations of hog kidney enzyme also catalyze the conversion of *N*-Ac-Gm-6-P to F-6-P and NH_3. In the present studies, acetokinase was used to show that acetate is an additional product of this reaction. Although 1360-fold purification of the hog kidney deaminase did not completely separate the enzymes which act on Gm-6-P and on *N*-Ac-Gm-6-P, the final step in the purification (freezing and thawing) inactivated the enzyme which reacts with *N*-Ac-Gm-6-P. The inactivated enzyme is postulated to be a deacylase which catalyzes Reaction V. A similar deacylase is present in many bacteria (34) and catalyzes the hydrolysis of *N*-Ac-Gm to Gm and acetate.[8] According to this hypothesis, the formation of F-6-P, NH_3, and acetate from *N*-Ac-Gm-6-P can be explained by the sum of Reactions IV and V. Attempts to demonstrate the formation of Gm-6-P in Reaction V, with use of crude hog kidney preparations, were unsuccessful owing to the activity of the deaminase.

The biosynthesis of glucosamine from glucose is known to proceed *in vivo* (35). *In vitro* studies demonstrated that the synthesis can proceed as indicated in Reaction VII with extracts from *N. crassa* (26, 36) and by an analogous reaction with extracts from rat liver (37). Since Gm-6-P can also be formed by the reversal of Reaction IV, conceivably this represents an additional pathway for the formation of Gm-6-P, despite the fact that IV proceeds primarily to the right. Thus, Reaction IV may serve to form

[8] *E. coli* extracts which hydrolyze the amide bond of *N*-Ac-Gm also hydrolyze the amide bond of *N*-Ac-Gm-6-P; whether one or two enzymes are involved is not yet known. These preliminary experiments were conducted by Mr. Jack Distler.

Gm-6-P from F-6-P and NH_3 by coupling it with Reaction VI. The enzyme which catalyzes Reaction VI is widely distributed in nature (14). In fact, *N*-Ac-Gm-6-P is rapidly formed *in vitro* by coupling the two reactions (Fig. 14). Whether similar reactions occur *in vivo* remains to be demonstrated.

SUMMARY

Glucosamine-6-phosphate (Gm-6-P) deaminase has been purified 120-fold from *Escherichia coli* and 1360-fold from hog kidney. Both enzymes catalyze the conversion of Gm-6-P to D-fructose 6-phosphate (F-6-P) and NH_3. Isotope and kinetic data indicate a single reaction mechanism, common to both enzymes, and that *N*-acetylglucosamine 6-phosphate (*N*-Ac-Gm-6-P) is not an intermediate in the reaction. *N*-Ac-Gm-6-P activates both enzymes but shows a more pronounced effect on the hog kidney deaminase.

The final purified preparation of each enzyme is specific for Gm-6-P and does not react with galactosamine 6-phosphate, *N*-acetylgalactosamine 6-phosphate, *N*-Ac-Gm-6-P, or combinations of these compounds.

The reactions are reversible, although F-6-P and NH_3 formation are greatly favored. However, by coupling the deaminase from either source with purified Gm-6-P acetylase, a rapid synthesis of *N*-Ac-Gm-6-P from F-6-P, NH_3, and acetyl coenzyme A was observed.

We are grateful to Mr. Jack Distler for the preparation of the labeled compounds and for his help with the isotope experiments. The advice of Dr. Jacob J. Blum with regard to the kinetic data is gratefully acknowledged.

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OBSERVATIONS ON THE FUNCTION OF SODIUM IN THE METABOLISM OF A MARINE BACTERIUM

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(Received for publication, January 16, 1958)

Bacteria have been found in the sea which are distinguishable from land and fresh water forms by having a highly specific and readily demonstrable requirement for Na^+ for growth (1). Like other bacteria investigated, they also require K^+. When Na^+ and K^+ were present in a medium at the concentrations of these ions in sea water, the concentration of Na^+ was found to be less and K^+ greater in the cells than in the medium. Furthermore, the quantitative requirements of the marine bacteria for Na^+ and K^+ proved to be very similar to those of animal cells in tissue culture. Marine bacterial cells and animal cells are thus very similar with regard to their relation to their inorganic environment, a point which may be of considerable significance from the standpoint of evolution.

Both Na^+ and K^+ have been found to be essential for the oxidation of exogenous substrates by cell suspensions of a marine bacterium (2). A more detailed investigation has shown that the requirement for Na^+, but not for K^+, varied according to the substrate being oxidized. Individual enzymes tested in cell-free extracts of the organism, on the other hand, did not require the addition of Na^+ for activity.

EXPERIMENTAL

The organism used was isolated from sea water and has been tentatively identified as a species of either a *Pseudomonas* or a *Spirillum*. The procedures for growing the organism and preparing cell suspensions have been described, and the medium used was only a slight modification of the one employed previously (2). To obtain cell suspensions capable of oxidizing malate, citrate, galactose, and glucose, cells had to be suitably adapted by incorporating the substrate to be oxidized into the growth medium. For this purpose the substrate was added at a 0.5 per cent level. Cells adapted to the oxidation of galactose could also oxidize glucose and were used for the glucose oxidation study. The cells were washed and suspended in a 0.052 M $MgCl_2$ solution for the purposes of this investigation. Oxidation of substrates by whole cell suspensions was followed manometrically by using a Warburg respirometer.

Cell-free extracts of the organisms were prepared by sonic disruption of the cells in a Raytheon 10 kc. sonic oscillator. Succinic dehydrogenase was assayed manometrically in the extract by measuring the rate of oxygen uptake in the presence of methylene blue (3). KCN was omitted when the effect of Na^+ and K^+ on the enzyme system was being determined. Fumarase was measured spectrophotometrically by observing the increase in optical density at 240 mμ with malate as substrate (4). Malic dehydrogenase activity was determined by following the rate of reduction of TPN (triphosphopyridine nucleotide) spectrophotometrically at 340 mμ (*cf.* (5)). For the cell-free extract studies tris(hydroxymethyl) aminomethane buffers were used throughout. Protein was estimated by the biuret method (6) with crystalline pepsin (Worthington Biochemical Corporation) as a standard.

Na^+ and K^+ determinations were carried out with the aid of a flame photometer attachment for a Beckman DU spectrophotometer equipped with a photomultiplier.

Results

With either acetate, butyrate, or propionate as substrate in the Warburg flask, a 50 mM solution of Na^+ was required for maximal rate of oxidation

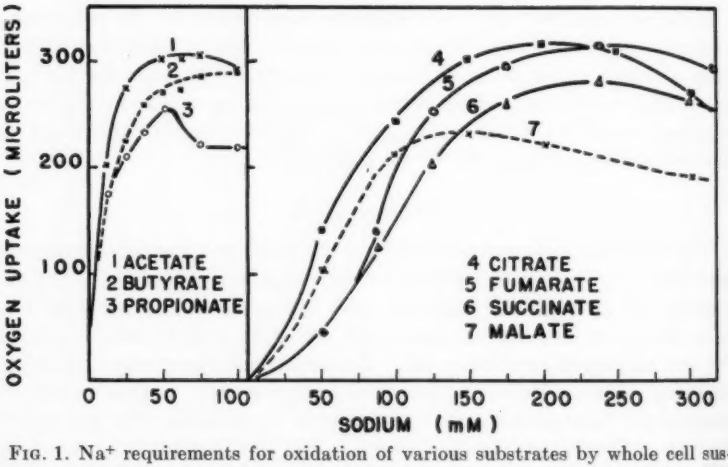

FIG. 1. Na^+ requirements for oxidation of various substrates by whole cell suspensions of a marine bacterium. Each Warburg vessel contained PO_4 buffer (K^+) 46 mM, pH 6.7, KCl 80 mM, $MgCl_2$ 3.4 mM, substrate (K^+ salt) 3.57 mM, 7 mg. dry weight of cells; total volume 2.8 ml.; center well 0.2 ml. of 20 per cent KOH; gas phase air; temperature 25°. Values recorded corrected for endogenous oxygen uptake at each level of Na^+ tested and represent oxygen uptake after the following incubation periods: fumarate 60 minutes, succinate and citrate 70 minutes, acetate, propionate, and butyrate 75 minutes, malate 90 minutes.

by the cell suspension (Fig. 1). For the oxidation of malate about three times and for that of citrate, fumarate, and succinate about four times, this level of Na^+ was required. When the requirements for K^+ for oxidation were determined, no variation with substrate could be detected (Fig. 2). The Na^+ and K^+ requirements for the oxidation of glucose and galactose by whole cell suspensions were determined and found to be almost identical with those found optimum for the monobasic acids.

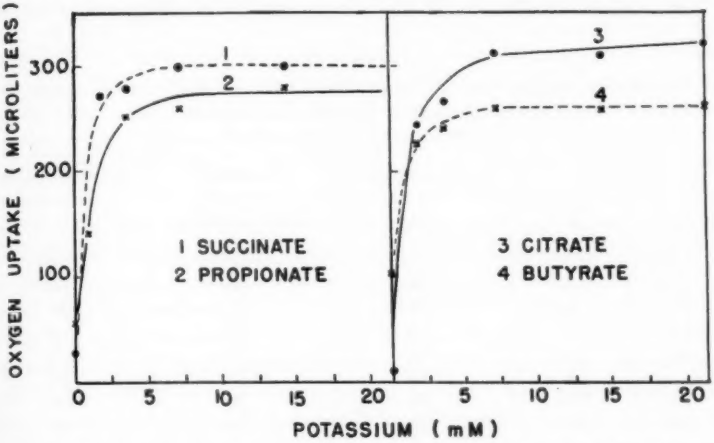

FIG. 2. K^+ requirements for oxidation of various substrates by whole cell suspensions of a marine bacterium. Each Warburg vessel contained tris(hydroxymethyl)-aminomethane buffer, adjusted to pH 6.7 with H_3PO_4, 46 mM, NaCl 77 mM for monobasic acids and 200 mM for di- and tribasic acids, $MgCl_2$ 3.4 mM, substrate (Na^+ salt) 3.57 mM, 7 mg. dry weight of cells; total volume 2.8 ml.; center well 0.2 ml. of 20 per cent KOH; gas phase air; temperature 25°. Values recorded corrected for endogenous oxygen uptake at each level of K^+ tested and represent oxygen uptake after the following incubation periods: citrate 55 minutes, succinate 75 minutes, butyrate 90 minutes, propionate 150 minutes.

Extracts of the organism were prepared by sonic disruption of the cells. Optimal conditions for the determination of the activity of succinic dehydrogenase, fumarase, and malic dehydrogenase in the extract were established. The relative activity of each enzyme in the presence of various concentrations of Na^+ and K^+ was then determined (Table I). Both succinic dehydrogenase and fumarase were found to be most active when neither Na^+ nor K^+ was added to the medium. In contrast to the activity of the other two enzyme systems studied, that of malic dehydrogenase was negligible unless K^+ was present. A 167 mM concentration of K^+ was found to produce an optimal response. In all cases, a level of

Na^+ and K^+ which would promote maximal rate of oxidation of the substrates by whole cell suspensions (200 mM Na^+ and 33 mM K^+) depressed enzyme activity in the cell-free extracts. Various other concentrations of Na^+ and K^+ tested had no additional stimulatory effect. Analyses for contaminating traces of the ions in those reaction systems which contained no added Na^+ and K^+ revealed that for the succinic dehydrogenase system the Na^+ and K^+ levels were 1.2 and 1.8 mM, for fumarase 0.02 and 0.026 mM, and for malic dehydrogenase 0.26 and 0.18 mM, respectively.

The results recorded in Table I for succinic dehydrogenase were obtained in a system containing no KCN to avoid adding K^+. With KCN present, however, the enzyme did not respond to added Na^+.

TABLE I

Activity of Succinic Dehydrogenase, Fumarase, and Malic Dehydrogenase in Cell-Free Extracts of Marine Bacterium at Various Concentrations of Na^+ and K^+

Ions added		Succinic dehydrogenase	Fumarase	Malic dehydrogenase
Na^+	K^+	Relative activity*		
mM	*mM*	*per cent*	*per cent*	*per cent*
0	0	100	100	8
200	33	65	25	37
200		55	36	5
33		88	97	8
	33	70	77	82
	200†	68	38	100
33	200†	58	30	100

* For each enzyme the maximal activity attained was assigned the value of 100. The activity under other conditions is expressed as per cent of the maximal activity.

† For malic dehydrogenase the level of K^+ for the optimal rate of oxidation (167 mM) was used.

DISCUSSION

The data presented reveal a quantitative requirement for Na^+ for the oxidation of substrates by whole cells which varied with the substrate tested, while the individual enzymes present in extracts of the cells showed no dependence on the presence of Na^+, at least in any appreciable amounts, for activity. To account for the large difference in Na^+ requirement for oxidation by whole cells of the monobasic acids and hexose sugars on the one hand and for the di- and tribasic acids on the other, one must conclude either that the two groups of compounds are being oxidized by

different oxidative pathways or that Na^+ is involved in the transport of at least some of the substrates across the cell membrane.

Considerable evidence that a tricarboxylic acid cycle exists in this organism has been obtained. Except for certain unusual features about the oxidation of propionate (7), there is no evidence to suggest that the monobasic acids are oxidized by other than this pathway. Since this metabolic pathway is cyclic in nature, a requirement for Na^+ at any step or for the transport of electrons to oxygen could not account for the differences in the Na^+ requirement of whole cells.

By the application of the nuclear spin resonance technique, evidence has been obtained indicating that Na^+ forms complexes with common metabolites (8). The strongest interactions with Na^+ have been found to occur among compounds forming chelates. The monobasic acids concerned here show little tendency to form chelates, while the di- and tribasic acids can form metal complexes (9). Differences in the Na^+ requirement for oxidation of the two groups of compounds can then best be accounted for by assuming that each of the di- and tribasic acids must form a complex with Na^+ before being transported across the cell membrane. The Na^+ requirement for the oxidation of the monobasic acids and monosaccharides would then be a reflection of the amount of Na^+ required intracellularly either for oxidative metabolism or to operate the Na^+ pump (10), while the additional amount needed for the oxidation of the di- and tribasic acids would represent the Na^+ required to form the various complexes. Such an explanation would also serve to account for Na^+ transport into the cell when appropriate substrates are present in the medium, but not when compounds which would not form associations with Na^+ are being oxidized.

Assays for the activity of many tricarboxylic acid cycle enzymes in animal tissue homogenates are carried out in the absence of added Na^+ or K^+ (11). Animal cells in tissue culture have also been shown to require Na^+ and K^+ for growth (12). It remains to be seen whether the phenomenon described here for marine bacterial cells occurs in animal tissues as well.

In having a requirement for K^+, the malic dehydrogenase of this organism resembles the malic enzymes of *Lactobacillus arabinosus* (13) and *Moraxella lwoffi* (14). It differs from them in being TPN-specific rather than diphosphopyridine nucleotide-specific and in the fact that the reaction with malate as substrate can be reversed by adding oxalacetate.

SUMMARY

With either acetate, butyrate, propionate, glucose, or galactose as substrate, cell suspensions of a marine bacterium required an approximately 50 mM concentration of Na^+ for maximal rate of oxidation. For the oxida-

tion of malate three times and for citrate, fumarate, and succinate about four times, this level of Na^+ was required. No variation in the K^+ requirement with different substrates could be detected.

In cell-free extracts of the organism, succinic dehydrogenase and fumarase were more active in the absence of added Na^+ and K^+ than in the presence of these ions. Malic dehydrogenase required the addition of K^+ for maximal activity. In all cases, levels of Na^+ and K^+ required for maximal rate of oxidation by cell suspensions inhibited the activity of the enzymes in the cell-free extracts.

It was concluded that the differences in the Na^+ requirement of cell suspensions with substrate observed could best be accounted for by assuming that the di- and tribasic acids, but not the monobasic acids and hexose sugars form complexes with Na^+ before crossing the cell membrane.

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ENZYMATIC SYNTHESIS OF *N*-GLUCOSYLURONIC ACID CONJUGATES*

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(Received for publication, December 11, 1957)

Enzymes in the microsomes of mammalian liver have been shown to transfer the glucuronosyl moiety from uridine diphosphate glucuronic acid to phenolic (2–4) and carboxylic acid acceptors (5). The present paper describes the enzymatic synthesis of another type of conjugate in which the glucuronic acid is transferred to an amino group.

Materials and Methods

Chemicals—*o*-Aminophenyl glucosiduronic acid was kindly supplied by Dr. R. T. Williams, St. Mary's Hospital Medical School, London. *o*-Aminobenzoyl and *p*-aminobenzoyl glucosiduronic acids were prepared enzymatically with UDP-glucuronic acid[1] and aglycone (5). Phenolphthalein glucosiduronic acid and bacterial β-glucuronidase were obtained from the Sigma Chemical Company, St. Louis, Missouri. UDP-glucuronic acid was prepared enzymatically from UDPG, with UDPG dehydrogenase and DPN (4).

An aniline-glucuronic acid complex was generously supplied by Dr. R. T. Williams (6). This compound contained 2 moles of aniline per mole of glucuronic acid. 1 mole of aniline could be removed by washing a solution of the compound at pH 8.0 with ether. The resulting aniline-glucuronic acid compound did not reduce triphenyltetrazolium in 0.5 N NaOH, but, following acidification with 0.1 N HCl, both the reducing group of glucuronic acid and the amino group of aniline were liberated. These observations indicated that the compound was *N*-phenylglucuronosylamine, in which the amino group was linked to carbon 1 of glucuronic acid.

Methods—Aniline and *p*-toluidine were extracted at pH 7.0 or above into 5 volumes of ethylene dichloride containing 1.5 per cent isoamyl alcohol. The compounds were returned to 1 ml. of 0.1 N HCl and determined in 0.5 ml. of the acid extract by diazotization and by coupling

* A preliminary report of this work has appeared previously (1).

[1] The following abbreviations will be used: DPN for oxidized diphosphopyridine nucleotide, UDP-glucuronic acid for uridine diphosphate glucuronic acid, UDPG for uridine diphosphate glucose, Tris for tris(hydroxymethyl)aminomethane.

with N 1-naphthylethylenediamine dihydrochloride (7). *p*-Phenetidine was assayed by a procedure described previously (8). In the determination of *N*-glucuronosyl compounds produced during the enzymatic reaction, the unchanged free amines were removed by shaking the incubation mixture twice with 5 volumes of ether. An equal volume of 0.5 N HCl was then added to the extracted aqueous solution. After waiting 5 minutes, the solution was made alkaline with sodium hydroxide, and the liberated amine was determined as described above.

o-Aminophenyl glucosiduronic acid was measured by the procedure of Levvy and Storey (9). *o*-Aminobenzoyl glucosiduronic acid and *p*-aminobenzoyl glucosiduronic acid were determined by the procedure described by Dutton (5). Phenolphthalein was measured at 540 mμ after adjusting the pH to 10.0. Glucuronic acid was estimated by the carbazole reaction (10).

Preparation of Tissues—Microsomes from guinea pig liver were prepared by a procedure described previously (4). Nuclei, mitochondria, and soluble fractions were prepared by differential centrifugation of liver homogenates in isotonic sucrose (11).

Enzyme Assay—A typical incubation consisted of microsomes obtained from 300 mg. of guinea pig liver, 25 μmoles of $MgCl_2$, 0.1 μmole of UDP-glucuronic acid, 0.5 μmole of substrate, 100 μmoles of Tris buffer, pH 8.0, and water to make a final volume of 1.5 ml. The mixture was incubated at 37° in a 15 ml. glass-stoppered centrifuge tube. After 30 minutes, the mixture was cooled, and the *N*-glucuronosyl derivatives were determined as described under "Methods."

Results

Enzymatic Synthesis of N-Glucuronosyl Derivatives of Aniline and Other Amines—The formation of a glucuronic acid conjugate of aniline was indicated by the disappearance of the amine after incubation with guinea pig microsomes and UDP-glucuronic acid (*cf.* (1), Table I). In the absence of UDP-glucuronic acid or when this cofactor was replaced with UDPG or glucuronolactone, little or no disappearance of aniline was observed. To determine whether the aniline that disappeared was present in "bound" form, the residual aniline was removed from the incubation mixture by extraction with ether, and the aqueous residue was acidified with hydrochloric acid. When this solution was kept at room temperature for 5 minutes, its extraction with ethylene dichloride at an alkaline pH was demonstrated by an almost complete recovery of the free amino compound. The liberated amine was identified as aniline by the technique of comparative distribution ratios (7). These observations suggested that the aniline which disappeared upon incubation with UDP-

glucuronic acid and guinea pig liver microsomes was converted to an acid-labile glucuronide, presumably *N*-phenylglucuronosylamine.

Further evidence for the identity of the enzymatically formed product was obtained by comparing its solubility and rate of hydrolysis with those of a synthetic sample. The biosynthetic and authentic samples in 0.1 N sodium hydroxide were shaken with 10 volumes of *n*-butanol (previously saturated with water), and the aqueous phase was assayed for conjugated aniline. 25 per cent of each compound was extracted into the organic solvent.

The rates of hydrolysis of the enzymatically formed and synthetic ani-

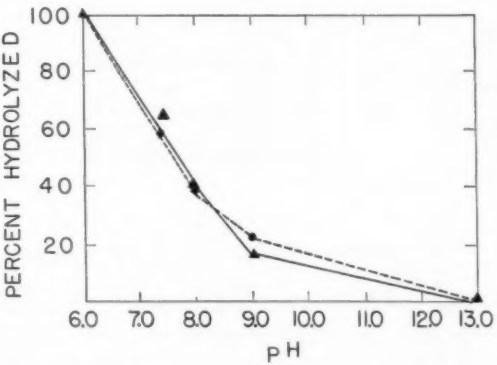

FIG. 1. Rates of hydrolysis of enzymatic (●) and synthetic (▲) *N*-phenylglucuronosylamine. Substrates (1×10^{-4} M) were incubated in 0.5 M Tris buffers at 37° for 60 minutes.

line conjugates were compared at various pH values and were found to be the same (Fig. 1). *N*-Phenylglucuronosylamine was most stable at an alkaline pH. Upon incubation with β-glucuronidase at pH 7.0, the rates of hydrolysis of both compounds were no greater than that observed with the boiled enzyme.

Incubating amines such as *p*-phenetidine and *p*-toluidine with guinea pig microsomes and UDP-glucuronic acid resulted in the formation of *N*-glucosyluronic acids (Table I).

Significant conjugating activity was present only in the microsomal fraction of guinea pig liver.

Properties of Enzyme System—In Tris buffer the rate of aniline conjugation was most rapid between pH 7.5 and 8.0. At pH 6.0 the reaction proceeded at 6 per cent of the maximal rate, while at pH 9.0 the reaction was 80 per cent of maximal.

As might be expected, the rates were the same whether incubation was aerobic or anaerobic.

The time-course of *N*-phenylglucuronosylamine formation is shown in

TABLE I

Enzymatic Synthesis of N-Glucosyluronic Acids

Washed microsomes obtained from 300 mg. of guinea pig liver were incubated at 37° with substrate, $MgCl_2$, and buffer, as described under "Methods." After incubation for 30 minutes, the reaction mixture was assayed for amine *N*-glucuronide.

Substrate	Additions (0.1 μmole each)	*N*-Glucosyluronic acid formed
		μmole
Aniline	None	0.008
"	UDP-glucuronic acid	0.060
p-Phenetidine	None	0.004
"	UDP-glucuronic acid	0.037
p-Toluidine	None	0.005
"	UDP-glucuronic acid	0.075

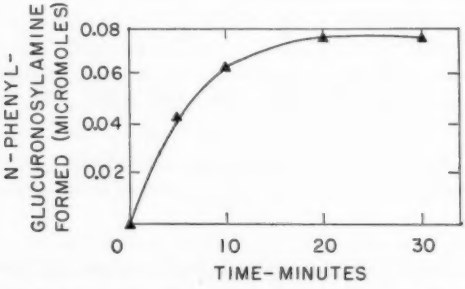

FIG. 2. Rate of *N*-phenylglucuronosylamine synthesis. Each sample contained microsomes from 300 mg. of guinea pig liver, aniline, and cofactors as described under "Methods."

Fig. 2. A similar rate of biosynthesis of hemiacetal and ester type glucuronide linkages has been shown (2, 5).

Table II shows that the presence of phenolic or carboxylic acid acceptors reduced the amount of *N*-phenylglucuronosylamine formed even in the presence of excessive amounts of UDP-glucuronic acid and that this depression could be overcome by adding larger amounts of the amino substrate.

Hydrolysis of Various Types of Glucuronide Linkages—The rates of hydrolysis of hemiacetal, ester, and *N*-glucosyluronic acids under dif-

ferent conditions were compared (Table III). *N*-Glucuronosyl derivatives were easily hydrolyzed in dilute acid at room temperature, but resisted attack by β-glucuronidase. The reverse was found to be true with hemi-

TABLE II

Inhibition of N-Glucosyluronic Acid Synthesis

Each sample containing microsomes obtained from 300 mg. of guinea pig liver, 100 μmoles of Tris buffer, pH 8.0, 25 μmoles of $MgCl_2$, 0.4 μmole of UDP-glucuronic acid, aniline hydrochloride, and inhibitors was incubated for 30 minutes at 37° and assayed for *N*-phenylglucuronosylamine formed.

Aniline (μmole)	Inhibitor (μmole)	Inhibition
		per cent
0.1	Phenol, 0.05	44
0.1	" 0.08	67
0.1	" 0.10	82
0.4	" 0.08	33
0.1	Benzoic acid, 0.1	13
0.1	" " 0.4	40
0.4	" " 0.1	0

TABLE III

Hydrolysis of Various Types of Glucuronic Acid Linkages

The final concentration of glucuronides was 1×10^{-4} M. The results are expressed as per cent of substrate hydrolyzed.

Conditions	*N*-Glucuronosyls of		*o*-Glucuronosides of		Ester glucuronides of	
	Aniline	*p*-Toluidine	*o*-Amino-phenol	Phenol-phthalein	*o*-Amino-benzoic acid	*p*-Amino-benzoic acid
β-Glucuronidase, 700 units, pH 7.4, 15 min., 37°	0*	0*	90	100	100	100
0.2 N HCl, 5 min., 25°	100	100	0	0	0	0
0.2 " NaOH, 20 hrs., 37°	30	50	0	0	100	75

* The rate of hydrolysis of *N*-glucosyluronic acids with β-glucuronidase was no greater than that observed with boiled enzyme.

acetal and ester type linkages. In alkaline solution, ester glucuronides were readily hydrolyzed, whereas hemiacetal glucuronides were stable. On the basis of these properties, the type of glucuronide linkage may be established.

Formation of N-Phenylglucuronosylamine in Vivo—Three guinea pigs were given 50 mg. per kilo of aniline hydrochloride intraperitoneally.

30 minutes before the administration of the amine, each animal received 600 mg. of sodium acetate to insure the excretion of an alkaline urine. Urine was collected for 24 hours in a beaker containing 2 ml. of 1 N sodium hydroxide. The free aniline was removed by extracting an aliquot of the urine with ethylene dichloride, and bound aniline was liberated after treatment with hydrochloric acid was determined. About 22 per cent of the administered aniline was excreted as an acid-labile conjugate. The conjugated aniline found in the urine had the same partition coefficient in the butanol-0.1 N NaOH system, as well as rates of hydrolysis at various pH values, as an authentic sample.

DISCUSSION

The data presented here demonstrate the enzymatic transfer of the glucuronosyl moiety of UDP-glucuronic acid to an amine acceptor. These observations suggest that this biosynthetic reaction may represent an important pathway for the metabolism of amino compounds and would explain the findings of others that the administration of amines results in the excretion of *N*-glucosyluronic acids (12, 13).

It appears likely, on the basis of the evidence presented by Dutton (5) and the current investigation, that a single enzyme catalyzes the transfer of glucuronic acid to phenolic, alcoholic, carboxylic, and amino acceptors, although a definitive answer must await purification of the enzyme. The failure of UDPG to act as a donor, however, indicates at least a certain degree of specificity with regard to the "active" sugar. Because of its non-specificity, the glucuronosyl-transferring enzyme appears eminently suitable for the "detoxification" of a wide variety of foreign compounds as well as of those which are normally present. It is interesting in this regard that, of the numerous possible alcoholic acceptors tested, all but *N*-acetylglucosamine inhibited the formation of *N*-phenylglucuronosylamine, which may suggest that *N*-acetylhyalobiuronic acid formation is not catalyzed by this enzyme.

The present study, taken with the work of other investigators, indicates that the formation of glucosiduronic acid is a nucleophilic substitution reaction in which the electron-donating aglycone displaces the UDP moiety (3) from the anomeric carbon atom of UDP-glucuronic acid by a backside attack. This formulation rationalizes the inversion of the α-glycosidic bond of the nucleotide to the β configuration of the glucosiduronic acid. On the basis of this mechanism, and in view of the lack of specificity of the transferring enzyme, one might predict that other nucleophilic substances, *e.g.* SH compounds, could also function as acceptors.[2]

[2] Recently Clapp (14) has isolated an *S*-glucuronosyl derivative from urine after the administration of a sulfonamide.

SUMMARY

An enzyme in the microsomes of guinea pig liver that can transfer glucuronic acid from uridine diphosphate glucuronic acid to the nitrogen group of amines is described. The possibility is considered that a single enzyme is involved in the synthesis of all types of glucosiduronic acid linkages.

Methods for the differentiation of hemiacetal, ester, and *N*-glucosyluronic acids are presented.

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FATTY ACID COMPOSITION OF COMPONENT LIPIDES FROM HUMAN PLASMA AND ATHEROMAS*

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(Received for publication, January 17, 1958)

The mounting evidence within the past few years that suggests the important role which dietary fats may play in renal-cardiovascular diseases has stimulated an unprecedented interest in almost every aspect of lipide metabolism, as indicated by the great increase in publications in this field. Unfortunately, owing to lack of satisfactory micro- or semimicro-methods for separating lipide components and determining their fatty acid composition, little has been published on the effect of dietary fatty acids on the fatty acid composition of the various body tissue lipides, although the need for these comprehensive studies has certainly been recognized.

Considerable attention is being given to the development of improved methods for the purpose, and recent advancements in lipide fractionation and analysis offer promise of useful application in lipide metabolism research: elution chromatography on silicic acid columns for separating extracted lipides into their components, *i.e.* sterol esters, glycerides, free sterols, and phospholipides (1–3); reverse phase partition chromatography for fractionating mg. quantities of fatty acids (4); gas liquid partition chromatography for analytical separation and determination of mg. quantities of fatty acids (5); and a spectrophotometric micromethod for determining polyunsaturated fatty acids in mg. sized samples (6).

These methods should still be considered in the developmental state, but, at least, attempts are being made to improve them. For example, Lipsky *et al.* (7) recently reported a modification of Borgström's silicic acid column technique, Crombie *et al.* (8) extended the reverse phase partition technique of Howard and Martin (4), apparatus for gas-liquid chromatography and techniques for its application to fatty acid analysis are still undergoing more or less continuous change and refinement; similarly, extensions and refinements have recently been proposed for the

* Presented at the meeting of the American Oil Chemists' Society, Cincinnati, Ohio, September 30 to October 2, 1957.

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spectrophotometric micromethod (9, 10). Although it seems probable that gas-liquid chromatography may ultimately be the method of choice for determination of fatty acids, the present status of its development leaves much to be desired as a routine quantitative method for determining unsaturated fatty acid components. Indeed, there is great need for more extensive experience in different laboratories with all of these more recent methods.

Very little has been published on the application of the recent advances in methodology for the separation of lipides of body tissues into their components and for the determination of fatty acid composition of each. Early studies on the nature of the fatty acids of cholesterol esters and other component lipides were limited to determination of the degree of unsaturation by means of iodine values (11–13). Recently, alkali isomerization spectrophotometric methods (14, 15) have been employed to determine the polyunsaturated acids of total lipides of human plasma. Mukherjee *et al.* (16) employed silicic acid chromatography to separate the component lipides of rat blood and a spectrophotometric method to determine the fatty acid composition of each. Freeman *et al.* (17) described a chromatographic method used in conjunction with infrared spectrophotometry to determine the proportions of lipide components in serum lipides, but did not report fatty acid analysis.

The present paper describes the separation of component lipides of human plasma and atheromas by means of silicic acid chromatography, and the determination of fatty acid composition of the component lipides by means of ultraviolet spectrophotometry and iodine numbers.

EXPERIMENTAL

Source of Tissue—The plasma was obtained by centrifuging whole blood from two healthy young male medical students (12 hours without food) for 1 hour at 2000 r.p.m. The plasma was pipetted from the cells and was first extracted with Delsal (18) solvent (4:1 methylal-methanol), and then the coagulated solids were given a final extraction with peroxide-free ethyl ether. After removal of the solvent, the crude lipide residues were reextracted with redistilled petroleum ether (b.p. 60–70°) and combined. From 415 ml. of plasma (corrected for added anticoagulant—acid-citrate-dextrose solution) 2.052 gm. of petroleum ether-soluble lipides were obtained.

The lipides from atheromas were obtained as follows. Atheromatous plaques, some ulcerated, were excised from human aorta taken at autopsy and extracted first with several portions of Delsal solvent and with ethyl ether. Then the tissue was finely shredded and reextracted by triturating in a mortar with successive portions of ethyl ether. After removal of

the solvents, the lipide residues were reextracted with petroleum ether (b.p. 60–70°) and combined. From 25 gm. of moist tissue, 4.35 gm. of petroleum ether-soluble lipides were obtained.

Solvents—All solvents used in any of the operations reported in this paper were carefully redistilled through a 24 inch Vigreux column before use. The ethyl ether employed as eluting solvent was treated with sodium and then distilled.

Separation of Lipides on Silicic Acid Column—An apparatus similar in design to that described in previous work (19, 20) for operation under slight but constant pressure of nitrogen (1 to 2 cm. of Hg above atmosphere) was used for separation of lipides. The glass column (21 × 300 mm.) was packed by introducing a mixture of 22.0 gm. of silicic acid[1]-filter aid[2] (80:20) in 100 ml. of petroleum ether (b.p. 35–60°). This mixture had been heated to boiling in a beaker, with stirring, for 5 minutes. During addition, slow stirring with a long thin stainless steel rod inserted in the column aided in uniformly packing the adsorbent as it settled by gravity. The adsorbent, when settled in the column, was about 190 mm. in height. The incorporation of a fraction cutter permitted collection of measured volumes of eluate under nitrogen.

About 100 mg. of weighed lipide in 10 ml. of petroleum ether were added by way of a separatory funnel connected to the top of the column. This was quantitatively washed down into the column with a wash of 10 ml. of the solvent, care being taken to keep the solvent level above the top of the adsorbent at all times. The first eluting solvent, 350 ml. of petroleum ether containing 1 per cent ethyl ether by volume, was then added to the separatory funnel, and the flow rate was adjusted to keep a constant level of several inches of liquid above the adsorbent. The rate of percolation about 150 ml. per hour was adjusted by increasing or decreasing the nitrogen pressure on the system. The order of elution, the same as that reported by others, was as follows: (1) hydrocarbons, 50 ml. of petroleum ether (+ 1 per cent ethyl ether); (2) sterol esters, 300 ml. of petroleum ether (+ 1 per cent ethyl ether); (3) glycerides, 300 ml. of petroleum ether (+ 4 per cent ethyl ether); (4) sterols + free acids, 350 ml. of petroleum ether (+ 8 per cent ethyl ether); and (5) phospholipides, 250 ml. of 25:75 methanol-ether followed by 250 ml. of 50:50 methanol-ether.

The separation of the plasma and atheroma lipides was similar except that no hydrocarbons were obtained from the atheroma lipides, and some

[1] Mallinckrodt's analytical grade (100 mesh), suitable for chromatographic analysis by the method of Ramsey and Patterson.

[2] Hyflo Super-Cel filter aid. (The mention of commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.)

of the phospholipides of the plasma were so strongly retained on the column that not all were removed. It was found more quantitative and practical to isolate them independently from the total lipides by precipitation with acetone and magnesium chloride, as described by Lipsky *et al.* (7).

Treatment and Analysis of Eluted Fractions—The solvent was removed from the eluted fractions under reduced pressure, and the lipide residues were transferred quantitatively to weighed (tared) 50 ml. round bottomed flasks with petroleum ether. This solvent was then removed, and the flask and contents were brought to dryness by the application of a vacuum and release to atmospheric pressure with nitrogen until constant weight was obtained. The fractions were saponified under nitrogen, the unsaponifiable material was extracted, and the fatty acids were recovered by quantitative extraction after acidification. The weighed fatty acids were made up to a standard volume with petroleum ether (b.p. 60–70°). Aliquots were taken for determination of polyunsaturated acids and iodine values.

Polyunsaturated Acids—The polyunsaturated acids were determined by a modification of the method described by Herb and Riemenschneider (6). Aliquots of the solution of weighed fatty acids obtained from the column separation (2.0 to 7.0 mg.) were added directly to the isomerization tube, and the solvent was removed under a stream of nitrogen and by warming. 2 gm. of 21 per cent KOH-glycol reagent were then added, and the sample was isomerized for 16 minutes at the usual 180°. The tubes were not removed from the bath during the period they were being shaken. (This modified procedure was tested along with the original procedure on numerous samples of different fats within the sample weight range of 1.0 to 7.0 mg., and the results showed good agreement.)

Iodine Value—Aliquots of the solution of weighed fatty acids representing 2.0 to 5.0 mg. of sample were added to 50 ml. glass-stoppered flasks. The solvent was removed under a stream of nitrogen and by warming. Chloroform, 0.4 ml., was added from a hypodermic syringe in such a manner as to wash down the wall of the flask. Exactly 1.0 ml. of 0.2 N Wijs solution was added with a micropipette. The flasks were then stoppered after the stoppers were slightly moistened with potassium iodide solution, and were stored in a dark cabinet for 20 minutes. After this reaction time, the stoppers were loosened enough to permit washing by dropwise addition of 0.4 ml. of 15 per cent KI solution to the flask, followed by the addition of about 4 ml. of distilled water. The iodine was titrated with 0.01 N sodium thiosulfate solution, with 4 to 8 drops of 1 per cent starch solution added as indicator.

RESULTS AND DISCUSSION

The separation of the sterol ester and glyceride fractions on the silicic acid column was essentially quantitative. Analyses of sterol ester frac-

tions from a number of experiments indicated that the sterol content of the ester fractions was from 59 to 62 per cent (theoretical recovery for cholesterol oleate, 59.5 per cent). Recovery of fatty acids from the saponified fractions was about 95 to 96 per cent of the theoretical. The glyceride fractions contained only minor amounts of sterol (1 to 5 per cent), which were removed after saponification and before recovery of the fatty acids. The free sterol and the free acids were eluted together in the same fraction. This fraction from the atheromas contained almost 97 per cent sterol, which had a melting point of 142° (micro melting point). The plasma sterol-acid fraction was usually about 80 per cent sterol. The acid portion of the fraction was confirmed by titrating with standard alkali by a microtechnique. These acids, however, were not isolated. The phospholipide

TABLE I

Component Lipides of Atheroma and Plasma

	Atheroma		Plasma	
	A	B	A	B
	per cent	*per cent*	*per cent*	*per cent*
Hydrocarbon			3.6	2.2
Cholesterol esters	27.8	28.1	39.3	41.3
Glycerides	20.4	18.4	14.3	13.8
Sterols	39.5	38.4	8.3	8.3
Fatty acids	Trace	Trace	2.3	2.2
Phospholipides	13.3	14.1	32.2	32.2

A and B represent duplicate determinations.

content of plasma lipides was obtained independently of the column separation. The small fraction isolated from the plasma before the cholesterol esters was identified by infrared analysis as hydrocarbon. The test for sterol in this fraction was negative. Table I summarizes the lipide composition of atheromas and plasma. Analyses for free and total cholesterol on the extracts (plasma-total cholesterol 31.5 per cent, free cholesterol 8.6 per cent; atheromas-total cholesterol 55.7 per cent, free cholesterol 39.5 per cent) agree very well with those derived from the fractionation data. The free sterols of the atheromas amounted to 70 per cent of the total sterols present, and the free sterols of the plasma were about 26 per cent of the total sterols of that tissue. The phospholipide content of the plasma was almost twice that of the atheromas, and the ratio of phospholipide to cholesterol was also much greater in the plasma than in the atheromas.

The fatty acid compositions of the fractions separated from atheromas and plasma lipides are compared in Tables II, III, and IV. The plasma fractions were almost twice as high in polyunsaturated acids as the cor-

responding fractions from atheromas. Over half of the polyunsaturated acids of both tissues was present as sterol esters. The oleic acid content of the fatty acids of cholesterol esters from atheromas was more than double

TABLE II

*Fatty Acid Composition of Sterol Esters**

Acid	Atheromas (iodine No. 134.0)	Plasma (iodine No. 142.7)
	per cent	*per cent*
Dienoic	23.62†	47.46‡
Trienoic	1.44	0.73
Tetraenoic	7.01	8.00
Pentaenoic	1.27	0.95
Hexaenoic	0.99	0.69
Oleic	61.50	23.49
Saturated	4.17	18.68

* Analysis conducted on fatty acids recovered after saponification and removal of unsaponifiable material.

† The value includes 2.28 per cent conjugated diene.

‡ The value includes 1.02 per cent conjugated diene.

TABLE III

*Fatty Acid Composition of Glycerides**

Acid	Atheromas (iodine No. 84.7)	Plasma (iodine No. 78.3)
	per cent	*per cent*
Dienoic	7.03†	14.27‡
Trienoic	0.52	1.34
Tetraenoic	2.26	1.26
Pentaenoic	0.60	0.47
Hexaenoic	0.00	0.54
Oleic	67.41	44.72
Saturated	22.18	37.40

* Analysis conducted on fatty acids recovered after saponification and removal of unsaponifiable material.

† The value includes 1.61 per cent conjugated diene.

‡ The value includes 2.12 per cent conjugated diene.

that from the plasma. It was also considerably greater in the glyceride and phospholipide fatty acids from atheromas. The saturated acids were somewhat higher in the plasma fractions. It is interesting to note that the cholesterol ester fatty acids of the atheromas contained only small amounts of saturated acids. Infrared spectroscopic examination of the fatty acids showed no measurable amount of trans double bonds.

The analyses of the total fatty acids of atheromas and plasma are presented in Table V. They, of course, reflect the fraction data and show

TABLE IV

*Fatty Acid Composition of Phospholipides**

Acid	Atheromas (iodine No. 77.1)	Plasma (iodine No. 101.8)
	per cent	*per cent*
Dienoic	7.21†	14.53‡
Trienoic	2.30	0.97
Tetraenoic	2.57	8.16
Pentaenoic	0.00	1.76
Hexaenoic	0.00	1.42
Oleic	55.70	35.50
Saturated	32.22	37.66

* Analysis conducted on fatty acids recovered after saponification and removal of unsaponifiable material.

† The value includes 2.93 per cent conjugated diene.

‡ The value includes 3.27 per cent conjugated diene.

TABLE V

*Fatty Acid Composition of Total Lipides**

Acid	Atheromas (iodine No. 110.0)	Plasma (iodine No. 113.0)
	per cent	*per cent*
Dienoic	15.99†	25.21‡
Trienoic	1.07	1.45
Tetraenoic	5.70	6.70
Pentaenoic	1.29	1.26
Hexaenoic	1.09	1.46
Oleic	54.40	32.51
Saturated	20.46	31.41

* Analysis conducted on fatty acids recovered after saponification and removal of unsaponifiable material.

† The value includes 1.68 per cent conjugated diene.

‡ The value includes 1.31 per cent conjugated diene.

the atheromas to be high in oleic acid but appreciably lower in both the saturated and polyunsaturated acids.

The fatty acid content of the fractions is of particular interest in the light of certain suggestions which have been made concerning the possible effect of saturated *versus* unsaturated acids on cholesterol atheromatous deposits. It has been theorized that a high saturated acid content of the diet may cause a preponderance of cholesterol esters of saturated acids in the blood.

These saturated acid sterol esters have a higher melting range and are less soluble and possibly less compatible in the blood than are the unsaturated esters. Thus, they may have a greater tendency to deposit in the coronary walls. If this is true, the cholesterol esters of the atheromas should have a higher saturated acid content than those of normal plasma. The results obtained do not support this idea. However, the difference in content of the various unsaturated acids warrants further investigation of the relation between plasma and atheromatous lipide compositions.

SUMMARY

Elution chromatography on silicic acid columns has been employed for the separation of tissue lipides into their component sterol esters, glycerides, free sterol-fatty acids, and phospholipides. A study was also made of the application of the spectrophotometric method for analysis of fatty acid composition of the lipide fractions. These procedures were successfully applied to the separation and analysis of lipides from human plasma and atheromas. The polyunsaturated acid content of the lipides from plasma was much greater than that for the corresponding lipides from atheromas, whereas the oleic acid content was much greater in the atheroma lipides. The saturated acid content of the cholesterol esters and glycerides from atheromas was considerably less than in corresponding fractions from plasma.

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DEOXYRIBOSYL COMPOUNDS IN ANIMAL TISSUES

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(Received for publication, January 22, 1958)

Several types of deoxyribosyl compounds have been found in the acid-soluble fraction of normal and malignant tissues (1–8). In 1955 Potter and Schlesinger (1) reported the isolation of deoxynucleoside mono-, di-, and triphosphates from extracts of calf thymus. More recently LePage (7) reported the isolation of deoxyadenosine triphosphate from a tumor. Schneider (2), using a microbiological assay method, found that small amounts of deoxyribosyl compounds were present in the blood and in all of the tissues of the rat and mouse which were examined. Studies on the isolation of the deoxyribosyl compounds of rat liver and blood indicated that deoxycytidine accounted for almost the entire microbiological activity of the tissue extracts (2, 3). Extracts of the Novikoff hepatoma and of regenerating liver, however, contained considerable proportions of deoxynucleotide-like material (3, 4).

Studies of the growth requirements of the organism used in the assay method (*Lactobacillus acidophilus* R-26) had indicated that equivalent growth was obtained with equimolar amounts of any of the natural deoxynucleosides or nucleoside monophosphates (2, 9, 10). Growth was also supported by dideoxynucleotides but not by deoxynucleoside di- or triphosphates (9). The present study was undertaken to develop methods whereby compounds of the latter type could be measured microbiologically. The following report describes methods for the enzymatic conversion of these compounds to nucleosides or nucleoside monophosphates and presents the results of simultaneous determinations of the content of deoxynucleosides, deoxynucleoside monophosphates, and "digestible" deoxynucleotides for various rat and mouse tissues. Those compounds which required enzymatic digestion for conversion to nucleosides or nucleoside monophosphates are designated as "digestible" nucleotides in this report.

Methods

Preparation of Tissue Extracts—Tissues[1] were removed as quickly as possible from animals under ether anesthesia and frozen in liquid nitrogen.

[1] The authors are indebted to Dr. A. J. Dalton and Dr. M. K. Barrett for some of the tumor transplants used in this study and to Miss Myra Embrey for performing the partial hepatectomies.

In some cases extracts were made of pooled tissues obtained from several animals and in others the frozen tissue was powdered and samples were taken for the preparation of the extracts. Usually the tissues were stored for 2 days or less at −20° before preparation of the extracts.

Samples of blood were removed by decapitating the animals and bleeding directly into liquid nitrogen. Urine was removed from the bladder by hypodermic syringe. Regenerating liver was obtained from rats by removing the median and left lateral lobes of the liver under ether anesthesia, according to the method of Higgins and Anderson (11). This tissue was frozen and stored for analysis as controls. At 24 and 48 hour intervals after the operation, the regenerated tissue was removed.

The frozen tissues were weighed and homogenized in a volume of ice-cold 0.6 N perchloric acid equal to 3 or 4 times the weight of the tissue. The homogenate was centrifuged for 30 minutes at 0–2° and 17,500 r.p.m. in the International model PR-1 centrifuge (rotor No. 291). The supernatant fluid was removed and neutralized to pH 7 with concentrated potassium hydroxide. Potassium perchlorate was removed by cooling to 0–2° and filtering.

Determination of Deoxyribosyl Compounds—The tissue extracts were assayed for their content of deoxyribosyl compounds according to a modification (2) of the method of Hoff-Jørgensen (10), with use of *L. acidophilus* R-26 (ATTC 11506). The deoxynucleoside content of the extracts was determined by assaying the effluent obtained by passing 3 ml. of the tissue extract through a Dowex 1-formate column, 5.0 × 0.9 cm. (prepared as described by Hurlbert *et al.* (12)), followed by 15 ml. of water. Under these conditions the nucleotides were retained on the column. The total deoxynucleoside plus deoxynucleoside monophosphate content of the tissue extracts was determined by assaying the extracts directly and the deoxynucleotide content was calculated by subtracting the nucleoside content.[2] In order to determine deoxynucleoside di- or triphosphates microbiologically, it was necessary to convert them to nucleosides or nucleoside monophosphates. The following three enzyme preparations were tested for this purpose: (1) a solution of lyophilized cobra venom; (2) potato apyrase, prepared as described by Lee and Eiler (13); and (3) an intestinal phosphatase preparation.[3] The venom preparation was unsuitable be-

[2] Since deoxynucleoside di- and triphosphates are partially converted to a microbiologically active form by autoclaving (9), high values for deoxynucleoside monophosphate content might be expected to result from autoclaving the tissue extracts with the culture medium. All of the extracts examined, with the exception of thymus, gave the same microbiological activity when sterilized by autoclaving or by filtration. The thymus extracts, however, gave higher values for deoxynucleotide when autoclaved and were consequently sterilized by filtration.

[3] We wish to thank Dr. R. Hilmoe and Dr. L. Heppel for supplying this enzyme preparation (No. 3-11-D52; activity, 7650 μmoles of phosphorus released per hour at

cause of its nucleosidase activity. The intestinal phosphatase, as will be reported below, gave higher values for digestible nucleotides than the potato enzyme, and was used in most of the work. The digestion mixtures consisted of 1 or 2 ml. of the tissue extract, 2 ml. of 0.1 M glycine buffer, pH 9, 0.2 ml. of 0.3 M magnesium acetate, and 0.05 ml. of intestinal phosphatase. After incubation for 1 hour at 37°, the digest was adjusted to approximately pH 7 and heated at 100° for 10 minutes. Aliquots of the digest were assayed microbiologically.

RESULTS AND DISCUSSION

Microbiological assay of the acid-soluble fraction of several rat and mouse tissues showed that small but variable amounts of all three types of deoxyribosyl compounds were present in all of the tissues studied (Table I). With the exception of spleen, thymus, and regenerating rat liver, the concentrations of nucleotides and digestible nucleotides, relative to the content of nucleosides, were greater in the case of the malignant tissues studied than in normal tissues. Since deoxyribonucleic acid (DNA) synthesis normally occurs at a high rate in spleen and thymus but not in liver or kidney (14), the data are compatible with the hypothesis that high deoxynucleotide levels are characteristic of growing tissues and may be closely related to DNA biosynthesis in these tissues. It will, of course, be necessary to investigate the turnover rates of the deoxynucleotides to determine their significance in DNA synthesis.

In regenerating liver as compared to normal liver, the relative concentration of digestible nucleotides was strikingly increased 24 hours postoperatively. The amounts of deoxynucleotides were also increased in the liver at this time but not as markedly as observed previously (4). The higher amounts reported earlier may be related to the fact (Table II) that the digestible nucleotide content decreased markedly on storage at −20° while the deoxynucleoside content increased correspondingly.

The fact that rat urine contained considerable amounts of deoxyribosyl material, most of which was deoxynucleoside, is of interest. The deoxynucleoside excreted is probably deoxycytidine since it has been shown that deoxycytidine accounts for all of the deoxyribosyl material in the blood ((2), and Table I). The experiments of Hecht and Potter (15) which led them to conclude that deoxycytidine was not a precursor of DNA in the liver during regeneration should be reevaluated from the standpoint of the circulating and excreted deoxynucleoside.

In order to determine the nature of the digestible deoxynucleotides, two types of studies were made. Investigation of the specificity of the intestinal phosphatase preparation showed that it exhibited diesterase,

37° per mg. of protein; prepared from a trypsin digest of intestinal mucosa by an unpublished procedure).

TABLE I

*Average Content of Deozyribosyl Compounds in Animal Tissues**

Species and strain		Age	Tissue	No. of analyses	Nucleosides	Nucleoside monophosphates	Increase in nucleotides after digestion†
		yrs.					
Mouse	C3H	1–2	Liver	2	0.4	0.2	0.4
		wks.					
	"	5–7	"	1	0.8	0.2	0.4
	"	5–7	Kidney	1	1.4	0.1	0.5
		yrs.					
	"	1–2	Spleen	1	7.1	8.0	7.3
		wks.					
	"	5–7	"	1	16.0	9.6	18.0
	"	5–7	Thymus	1	15.0	19.1	19.3
	DBA		Thymoma Dalton	2	9.6 (8.4–10.8)	6.5 (6.4–6.5)	7.1 (6.9–7.3)
	C3H		Adenocarcinoma C3HBA	2	4.4 (4.1–4.7)	2.5 (2.4–2.6)	4.4 (3.9–4.8)
	"		Hepatoma 98/15	2	1.9 (1.8–2.0)	1.1	4.7 (4.6–4.7)
Rat	Sprague-Dawley		Blood	6	11 (10–13)	1	0 (0–2)
	Sprague-Dawley		Urine	1	29	0	5
	Sprague-Dawley		Liver	16	9 (6–14)	1 (1–2)	3 (2–5)
	Sprague-Dawley		Kidney	4	14 (12–18)	1	2 (1–2)
	Sprague-Dawley		Lung	4	9 (8–9)	2 (1–3)	2
	Sprague-Dawley		Spleen	4	19 (18–19)	7 (5–9)	2 (1–3)
	Sprague-Dawley		Thymus	11	27 (24–37)	20‡ (14–26)	36 (25–46)
	Sprague-Dawley		Regenerating liver, 24 hr.	8	9 (6–12)	8 (4–7)	13 (8–21)
	Sprague-Dawley		Regenerating liver, 48 hr.	14	11 (8–14)	2 (0–4)	8 (1–11)
	Holtzman		Novikoff hepatoma	12	7 (4–10)	5 (2–6)	6 (3–8)

* Expressed as micrograms of thymidine per gm. of frozen tissue or per ml. of urine. The numbers in parentheses indicate ranges.

† Intestinal phosphatase.

‡ All tissue extracts were sterilized by autoclaving with the exception of the extracts of thymus which were sterilized by filtration through an ultrafine fritted glass filter.

adenosinetriphosphatase, and diphosphopyridine nucleotidase activities in addition to its phosphomonoesterase activity. The digestible nucleotides could thus consist of compounds other than the nucleoside polyphosphates isolated by Potter *et al.* (1, 5) and LePage (7). This was also

TABLE II

*Changes in Content of Deoxyribosyl Compounds during Storage of 24 Hour Regenerating Liver at −20°**

No. of days stored	Nucleosides	Nucleoside monophosphates	Digestible nucleotides
2	10.4	5.6	10.1
22	13.9	7.7	2.7
49	17.6	4.5	1.2

* Expressed as micrograms of thymidine per gm. of frozen tissue.

TABLE III

*Tissue Content of Digestible Deoxynucleotides Determined after Intestinal Phosphatase or Potato Apyrase Digestion**

Species and tissue		Intestinal phosphatase	Potato apyrase
Mouse	Liver	0.4	0.1
	Spleen	18.0	9.1
	Thymus	19.3	10.0
	Kidney	0.5	0.1
	Thymoma Dalton	7.1	1.7
	Adenocarcinoma C3HBA	4.4	1.0
	Hepatoma 98/15	4.7	0.6
Rat	Lung	1.9	0.6
	Spleen	7.2	2.4
	Thymus	35.9	27.4
	Regenerating liver, 48 hr.	7.0	1.0
	Novikoff hepatoma	10.8	2.4

* Expressed as micrograms of thymidine per gm. of frozen tissue.

indicated by experiments in which the amounts of digestible nucleotides were determined with either intestinal phosphatase or potato apyrase. The results (Table III) show that larger amounts of digestible deoxynucleotides were consistently obtained after phosphatase digestion than after apyrase digestion. Two compounds that are attacked by diphosphopyridine nucleotidase, but not by the apyrase, are deoxycytidine diphosphoethanolamine and deoxycytidine diphosphocholine. These compounds have been isolated from extracts of thymus (6) and *Arbacia*

eggs (8), respectively. We have independently isolated from the Novikoff hepatoma a compound which is similar in some respects to deoxycytidine diphosphoethanolamine. This compound appears to account for about 25 per cent of the hepatoma digestible deoxynucleotide and will be described in a subsequent report.

SUMMARY

The concentrations of deoxynucleosides, deoxynucleoside monophosphates, and "digestible" deoxynucleotides in tissues were determined for a number of normal and malignant tissues of the rat and mouse. The designation, "digestible" deoxynucleotides, refers to nucleotides which require enzymatic digestion for conversion to nucleotide monophosphates or nucleosides. It appears that, with some exceptions, the levels of the nucleotides and digestible nucleotides are higher in malignant than in normal tissue, relative to the concentration of nucleosides, and are relatively higher in regenerating liver, 24 hours postoperatively, than in the controls. The data suggest that this difference may be characteristic of growing tissue.

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ASSAY OF TISSUE PHOSPHOGLUCOMUTASE ACTIVITY*

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(Received for publication, January 2, 1958)

Although several values for tissue phosphoglucomutase activites are recorded in the literature, such determinations have been performed without the addition of the coenzyme, α-glucose 1,6-diphosphate (1–3), and occasionally without explicit designation of the optimal concentrations of either or both of the known activators, Mg^{++} and amino acid (2, 3). Although some of these latter factors have been previously considered (4), the present paper is concerned with the extent to which added coenzyme influences the mutase activity of tissue homogenates and the conditions under which coenzyme and the other activating compounds give maximal phosphoglucomutase activity and hence yield a precise method of assay for this enzyme in tissue.

EXPERIMENTAL

Whole liver or samples of the gastrocnemius muscle of the rat were homogenized immediately after removal in a Waring blendor in the cold room with 50 to 100 volumes of distilled water. Human tissue was removed at autopsy or biopsy, placed into the deep freeze, and homogenized within 1 to 5 days. Further dilutions were made as required for specific experiments. Crystalline phosphoglucomutase was prepared from rabbit muscle according to Najjar's method (5), dissolved in 0.15 M acetate buffer at pH 5, and kept in the deep freeze. The protein concentration was 6.7 mg. per cc. Two preparations of the coenzyme, α-glucose 1,6-diphosphate, were used in the present work.[1] The first was prepared by Posternak (6) and obtained by us in November, 1949, through the courtesy of Dr. E. W. Sutherland. The second preparation was obtained in June, 1956, from Dr. L. F. Leloir through the late Dr. E. S. G. Barron. These are desig-

* This work has been supported in part by American Cancer Society grants, No. T-2 and No. T-18 and by grant No. DRG-332B from the Damon Runyon Memorial Fund for Cancer Research.

[1] The coenzyme preparations were dissolved to make solutions of the order of 1 to 2×10^{-4} M, and further dilutions were made as necessary. Several experiments in which aliquots of the dissolved coenzyme were treated with Na_2SO_4 to remove the Ba showed no significant differences between the dissolved Ba salt and the converted coenzyme with regard to the degree of activation of crystalline or liver homogenate phosphoglucomutase.

nated in the text and in Tables I to IV as Preparations P and L, respectively.

Phosphoglucomutase activity was measured by the conversion of the acid-labile phosphorus of glucose 1-phosphate (G-1-P) into the acid-stable phosphorus of glucose 6-phosphate (G-6-P) in accordance with methods previously described (4, 7, 8). 0.5 cc. of tissue homogenate, or appropriate dilution of the crystalline enzyme, was added to 0.5 cc. of 0.025 M glucose 1-phosphate (dipotassium salt, Schwarz) adjusted to pH 7.6 ± 0.05, 1.0 cc. of 0.1 M tris(hydroxymethyl)aminomethane buffer (Matheson) at pH 7.5, and 0.5 cc. of solutions of other compounds, as noted in connection with specific experiments. The final concentration of substrate was 0.005 M, equivalent to the formation of 155 γ of G-6-P phosphorus per cc. of the reaction mixture. The temperature of the reaction was 37°.

Results

Effect of Mg^{++} and Amino Acid—Table I shows a typical experiment in which the addition of 0.6×10^{-3} to 1.2×10^{-3} M Mg^{++} provided the optimal concentration for activity of tissue phosphoglucomutase at optimal pH and in the presence of 2×10^{-2} M L-histidine or 8×10^{-3} M L-cysteine and of coenzyme. Added concentrations of 2.5×10^{-3} M Mg^{++} or higher caused inhibition. The range, 0.6×10^{-3} to 1.2×10^{-3} M Mg^{++}, was also found to be optimal for human muscle homogenate. The concentrations of L-histidine and L-cysteine necessary for optimal activity of rat liver homogenates were, respectively, 2×10^{-2} and 8×10^{-3} M (Table II). The optimal concentrations of Mg^{++} and amino acids were slightly less than those required for optimal activation of crystalline phosphoglucomutase (5, 7, 9). The ratio of the optimal activity in the presence of histidine to that in the presence of cysteine was 0.76, essentially the same as that reported for crystalline phosphoglucomutase (10).

Effect of pH and Concentration of Tissue Homogenate on Phosphoglucomutase Activity—The pH necessary for optimal activity of rat liver homogenates in the presence of 1.2×10^{-3} M Mg^{++}, 2×10^{-2} M histidine, and 1.2×10^{-6} M α-glucose 1,6 diphosphate was found to be, as in the case of rabbit muscle extracts (8) and crystalline preparations of phosphoglucomutase, between pH 7.5 and 7.6 (5, 9). The time-course of the reaction in liver homogenates, in the presence of added Mg^{++} and amino acid and with or without added coenzyme, was of zero order for about the first 70 per cent conversion of the substrate, as has also been observed with crystalline preparations of the enzyme (5, 7). When the substrate change during the zero order portion of the reaction was used as a measure of reaction velocity, the enzyme activity was directly proportional to the concentration of homogenate in the presence of optimal concentrations of activating amino

acid and Mg^{++} and either in the presence or absence of an added final concentration of 1.2×10^{-6} M α-glucose 1,6-diphosphate (Fig. 1).

TABLE I

Effect of Magnesium on Phosphoglucomutase Activity of Rat Liver Homogenates

Final concentrations of liver homogenate, 1:500; of added α-glucose 1,6-diphosphate (Preparation P), 1.2×10^{-6} M; time of reaction, 30 minutes. Other conditions are as stated in the text.

Added concentration of Mg^{++}	Rate of formation of G-6-P phosphorus in presence of	
	2×10^{-3} M L-histidine	8×10^{-3} M L-cysteine
mM	*γ per cc.*	*γ per cc.*
0.0	12	19
0.6	51	71
1.2	56	69
2.4	40	58
4.8	26	36

TABLE II

Effect of L-Histidine and L-Cysteine on Phosphoglucomutase Activity of Rat Liver Homogenates

Final concentrations of liver homogenate, 1:625; of added Mg^{++}, 1.2×10^{-3} M; of added α-glucose 1,6-diphosphate (Preparation P), 1.2×10^{-6} M. Time of reaction, 30 minutes. Other conditions are as stated in the text.

Added concentration of amino acid	Rate of formation of G-6-P phosphorus in presence of	
	L-Histidine	L-Cysteine
mM	*γ per cc.*	*γ per cc.*
0	0	0
2	15	21
4	20	51
8	33	62
10	38	61
20	47	59
40	38	44

Influence of Concentration of Coenzyme, α-Glucose 1,6-Diphosphate, on Tissue Phosphoglucomutase Activity—Sutherland and his associates (11) indicated that a final concentration of about 2.0×10^{-6} M α-glucose 1,6-diphosphate produced maximal activation of crystalline rabbit muscle phosphoglucomutase in the presence of optimal concentrations of Mg^{++} and amino acid. Cardini *et al.* (12) found that at 0.8×10^{-9} mole of coenzyme

in 0.3 cc. of reaction mixture, or a concentration of 2.7×10^{-6} M, the velocities were approaching but had not attained a maximum. In the present study, several preliminary experiments on human serum and rat liver and human muscle homogenates showed that the reaction velocities did not reach a maximal value at concentrations as high as 6×10^{-6} M to 8×10^{-6} M of Preparation P of coenzyme. Because of the possibility that coenzyme was being destroyed by the serum or tissue, its effect was reevaluated on crystalline phosphoglucomutase. In several series of experiments, with concentrations of enzyme protein ranging from 0.9 to 4.4 γ per cc. of the reaction mixture and final added concentrations of 1.2×10^{-3} M Mg^{++} and 8×10^{-3} M cysteine, maximal reaction velocities were virtually reached at added concentrations above 10×10^{-6} M with the Preparation P and above 4×10^{-6} M with the more recently obtained Preparation L of coenzyme. Extrapolation of the reaction velocity-coenzyme concentration curves to zero reaction velocity showed that the substrate, G-1-P, contributed about 0.3×10^{-6} M coenzyme concentration. The findings that the Preparation P of coenzyme was considerably less active than Preparation L and that much higher concentrations were necessary for activation of crystalline phosphoglucomutase than had been reported in 1949, indicated that Preparation P, though kept in the refrigerator and deep freeze, had lost activity during the intervening years.

The necessity for using high concentrations of the coenzyme preparations to attain maximal activity and the general scarcity of this compound made it desirable to devise a method for calculating reaction velocity at infinite coenzyme concentration from determinations at relatively low coenzyme concentrations. The relationship between enzyme reaction velocity and coenzyme concentration, $K = [(v_{max} - v)/v] \times c$, proposed by Sutherland and coworkers (11), may be transformed into a linear form, $1/v = K/v_{max} \times 1/c + 1/v_{max}$, where v is the reaction velocity at coenzyme concentration, c. The reaction velocities obtained in several series of experiments with varying dilutions of crystalline phosphoglucomutase and varying concentrations of coenzyme were converted to a common basis, the amount of G-6-P phosphorus formed per cc. of the reaction mixture in 5 minutes by an enzyme concentration of 1.3 γ of enzyme protein per cc. of the reaction mixture. The reciprocals of these velocities were plotted against the reciprocals of the coenzyme concentration (Fig. 2). The results yielded a straight line in agreement with the formulation of Sutherland and associates (11). Although the slopes of the lines for Preparations P and L were different, the intercepts at $1/c = 0$ were the same, namely $1/v \times 10^3 = 17$. This method of plotting enables the calculation of maximal reaction velocity at infinite coenzyme concentration, and this value is independent of the particular preparation of coenzyme employed.

Assay of Tissue Phosphoglucomutase Activity—The determination of this enzyme activity in homogenates in the absence of added coenzyme may reflect variability in the coenzyme content of the tissue (12) and substrate (11). The determination of the activity at infinite coenzyme concentration, and at optimal concentrations of Mg^{++} and amino acid, provides a standard condition for the measurement of the enzyme concentration in tissue. When the reciprocals of the velocities obtained with homogenates

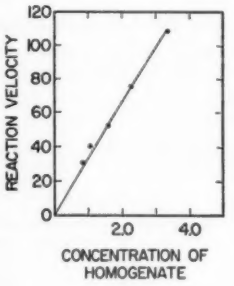

FIG. 1

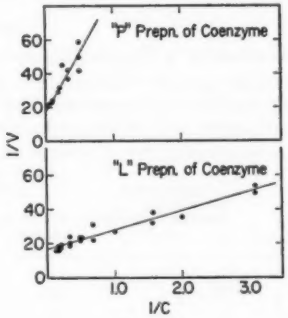

FIG. 2

FIG. 1. Direct proportionality between velocity, expressed as micrograms of G-6-P phosphorus formed per cc. of the reaction mixture, and concentration of rat liver, expressed as mg. of tissue per cc. of the reaction mixture. Final concentrations of added Mg^{++}, 1.2×10^{-2} M; of added cysteine, 8×10^{-3} M; and of added α-glucose 1,6 diphosphate, 1.2×10^{-6} M. Temperature, 37°; pH in optimal range, 7.5 to 7.6.

FIG. 2. Reciprocal plot of the effect of the concentration of coenzyme, α-glucose 1,6-diphosphate, on crystalline phosphoglucomutase activity. As noted in the text, P refers to the Posternak preparation of coenzyme and L to the Leloir preparation. Velocity (v) is expressed as mg. of G-6-P phosphorus formed in 5 minutes per cc. of the reaction mixture. The concentration of coenzyme (c) is expressed as micromoles per liter of the reaction mixture. Final concentrations of Mg^{++}, 1.2×10^{-2} M; of L-cysteine, 8×10^{-3} M. Temperature, 37°; pH in optimal range, 7.5 to 7.6.

of liver and muscle were plotted against the reciprocals of the coenzyme concentrations, straight line relationships also resulted, in agreement with the equation, $1/v = K/v_{max} \times 1/c + 1/v_{max}$. Although the slope might occasionally differ, depending upon whether c represented the concentration of coenzyme added to the reaction mixture or the sum of that added and the concentrations in substrate and tissue, the intercept on the axis of ordinates, namely the value for $1/v_{max}$ and therefore v_{max}, was not affected.

In accordance with the preceding considerations, the determination of the reaction velocities at two relatively low concentrations of coenzyme preparation, the drawing of a straight line through the corresponding co-

ordinates, and the determination of the intercept on the axis of ordinates at $1/c = 0$, permit the estimation of maximal phosphoglucomutase activity in tissues. Table III shows the application of this procedure to the determination of this enzyme activity in rat liver and skeletal muscle. The average of ratios of activity with histidine to those with cysteine was 0.75. The values obtained in the presence of L-histidine multiplied by 1.33 were therefore equivalent to values obtainable with L-cysteine. The activities

TABLE III

*Determination of Phosphoglucomutase Activity of Rat Liver and Muscle**

Final concentrations of added Mg^{++}, 1.2×10^{-3} M; of added L-cysteine, 8×10^{-3} M. Preparation P of coenzyme was employed; pH, 7.5 to 7.6; time of reaction, 0.5 hour; temperature, 37°. Male animals were used.

Tissue	Concentration of tissue per cc. reaction mixture	G-6-P phosphorus formed per cc. at added coenzyme concentration of			G-6-P phosphorus formed per cc. in 1 hr. by 100 mg. tissue
		1.2 μM	2.0 μM	∞ (Calculated)	
	mg.	γ	γ	γ	mg.
Liver 1	1.6	60	76	111	14.0
" 2	1.6	64	85	194	24.0
" 3	1.6	34	52	166	20.7
" 4	1.6	64	80	129	16.2
" 5	1.6	53	65	111	13.8
Muscle 1	1.16	77	96	152	26.3
" 2	1.00	59	80	152	30.4

* Glucose-6-phosphatase activity in the liver homogenates, as manifested by the liberation of inorganic phosphate during the reaction, was negligible. The removal of glucose-6-phosphatase by the procedure of Cori and Schulman (1) was also found not to affect the extent of phosphoglucomutase action and hence was not employed in the above analyses.

were finally calculated as the mg. of phosphorus in G-1-P converted to G-6-P by 100 mg. of liver in 1 hour.

Comparison of the values for phosphoglucomutase activities obtained by the procedure described in this paper with those in the literature is difficult because there are so few previously recorded values on similar tissues. Dreyfus and Schapira (2), by employing a reaction mixture containing final concentrations of 0.005 M G-1-P, 0.025 M added cysteine, and 1.5×10^{-3} M added Mg^{++} but no added coenzyme, found the mutase activity of four control human muscle specimens to range from 13 to 17.5 mg. of P and to average 15 mg. of P transformed from G-1-P into G-6-P in 15 minutes at 37° per gm. of human muscle. This is equivalent to a change of 6.0 mg. of P in 1 hour by 100 mg. of tissue at 37°, the units used in the

present study. Table IV shows that the average of values from our analyses on muscle from two patients with cancer and one with chronic myositis was about 16 mg. of P. These values were two to three times as high as the control values reported by Dreyfus and Schapira (2) and about six to nine times as high as those from patients with various muscular diseases.

The author wishes to thank Dr. Marie N. Lipsett for the preparation of the crystalline phosphoglucomutase and Mrs. Joyce London for her technical assistance.

TABLE IV

Determination of Phosphoglucomutase Activity of Human Muscle

Final concentrations of added Mg^{++}, 1.2×10^{-3} M; of added amino acid, 8×10^{-3} M L-cysteine, or 2×10^{-2} M L-histidine; time of reaction, 1.0 hour; temperature, 37°; Preparation P of coenzyme.

Specimen No.	Concentration of tissue per cc. reaction mixture	Amino acid	G-6-P phosphorus formed per cc. at added coenzyme concentration of				G-6-P phosphorus formed per cc. in 1 hr. by 100 mg. tissue*
			0 μM	1.2 μM	2.0 μM	∞ (Calculated)	
	mg.		γ	γ	γ	γ	mg.
1	1.0	L-Cysteine		49	66	139	13.9
2	0.8	L-Histidine	38	78	93†	128	21.3
3	0.8	"	24	39	51†	85	14.1

* These values represent activities at optimal L-cysteine concentration; the activities of Specimens 2 and 3, obtained with L-histidine, were multiplied by 1.33.

† These values were interpolated from velocity-coenzyme concentrations curves.

SUMMARY

A method is proposed for the assay of phosphoglucomutase activity in tissue based on the determination of this enzyme activity in homogenates at 37°, at an optimal pH of 7.5 to 7.6, optimal concentrations of 1.2×10^{-3} M added Mg^{++}, of 8×10^{-3} M added L-cysteine, and at a calculated infinite concentration of the coenzyme, α-glucose 1,6-diphosphate. The optimal concentrations of Mg^{++} and L-cysteine should be confirmed for tissues other than those noted in the present paper.

The addition of coenzyme increases considerably the phosphoglucomutase activity of tissue homogenates. The determination of the reaction velocity at a calculated, infinite concentration of the coenzyme provides a standardized condition for the assay, is independent of the purity of the coenzyme preparation, and obviates the use of the large amounts of coenzyme, at present in short supply, that would actually be needed to elicit maximal phosphoglucomutase activity in tissue assays.

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THE RESISTANCE OF α-AMYLASES TOWARDS PROTEOLYTIC ATTACK*

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(Received for publication, February 3, 1958)

It has often been reported that α-amylases become increasingly unstable in the course of purification (2–4). Thus, Meyer *et al.* noted that partially purified solutions of amylase from hog pancreas could lose up to 75 per cent of activity in 24 hours at 0° and 80 per cent in 0.5 hour when kept at room temperature, and that the rate of inactivation was increased by dialysis. The inactive material could be crystallized and was shown to consist of breakdown products of the original enzyme (5). Stabilization of hog pancreas amylase was achieved by addition of a boiled amylase solution, and this made possible the final purification and crystallization of the active enzyme (3), which proved to be quite stable after two additional crystallizations (6).

To explain these observations, it was proposed (3, 5) that (*a*) α-amylases could undergo a reversible dissociation into an inactive apoenzyme and a dialyzable, thermostable cofactor, presumed to be present in the boiled amylase solution; (*b*) in a second reaction, the free apoenzyme could be broken down by traces of proteolytic contaminants which are carried along in the preparation, at least until the enzyme is crystallized; (*c*) addition of the cofactor to the apoenzyme could prevent its proteolysis, presumably by reforming the resistant, active holoenzyme. These reactions were represented as follows

$$\text{Active enzyme} \rightleftharpoons \text{apoenzyme} + \text{cofactor}$$
$$\downarrow \text{ proteolysis}$$
$$\text{breakdown products}$$

Although the nature of the stabilizing factor present in boiled amylase solutions was never clearly established, this material was shown to contain small amounts of Ca^{++}. Further investigations indicated that the addition of Ca^{++} salts alone, at various stages of the purification, could result in an appreciable stabilization of the enzyme (7), whereas sequestering agents, in contrast, caused a rapid inactivation. These studies were

* A preliminary report of this work was presented before the Forty-eighth annual meeting of the American Society of Biological Chemists at Chicago, Apr. 15–19, 1957 (1).

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extended by other investigators to amylases from bacterial and mold origin; their results showed that calcium is involved in the protection of the enzyme from denaturation and proteolytic degradation (8–10).

The purpose of the present work was to determine the circumstances under which amylases can resist proteolysis. This is of obvious importance in view of the fact that in many cases these enzymes are required to function physiologically in the presence of high concentrations of proteases (pancreatic juice, bacterial and mold cultures, etc.). In order to determine whether the effects observed are general attributes of α-amylases, this study was carried out on crystalline α-amylases from widely different origin; namely, human saliva, swine pancreas, *Bacillus subtilis*, and *Aspergillus oryzae*.

EXPERIMENTAL

Materials and Methods

Unless otherwise stated, *α-amylases* from human saliva, hog pancreas, *B. subtilis*, and *A. oryzae* were purified and crystallized according to established procedures (7). *Trypsin* was purchased from the Worthington Biochemical Corporation, Freehold, New Jersey, as a twice crystallized material containing approximately 50 per cent $MgSO_4$. *α-Chymotrypsin* was a three times crystallized preparation kindly made available by Dr. Hans Neurath. Both proteases were dialyzed overnight in the cold against dilute HCl, pH 3.0, before use. *Soy bean trypsin inhibitor* was purchased from the Worthington Biochemical Corporation and was used as a solution in GP[1] buffer, pH 7.5.

The concentration of the enzymes was determined by ultraviolet absorption spectra (Beckman DU spectrophotometer), the following values being used for 1 per cent solutions of protein: $E_{280}^{1\text{ cm.}} = 26$ for human saliva and hog pancreas α-amylases, 25 for *B. subtilis* α-amylase, and 20 for *A. oryzae* α-amylase as determined in this laboratory, 14.4 for trypsin (11), and 20 for chymotrypsin (12). For the calculation of molar ratios between the various components of the systems studied, the following molecular weights were used: 50,000 for the amylases, 23,000 for trypsin, 25,000 for chymotrypsin, and 18,000 for soy bean trypsin inhibitor.

DFP was synthesized according to the procedure of Saunders and Stacey (13), and 10^{-1} M and 10^{-2} M stock solutions were prepared in dry isopropanol. EDTA (Baker Chemical Company) and all other chemicals were reagent grade. Disodium glycerophosphate (lot No. 644, Eastman Organic Chem-

[1] The following abbreviations are used in this paper: EDTA, sodium ethylenediaminetetraacetate; DFP, diisopropylphosphofluoridate; GP, sodium glycerophosphate.

icals Department, Division of the Eastman Kodak Company) was used in preparing the buffers.

Determination of Enzyme Activity

α-Amylases were determined according to a modification of the dinitrosalicylic acid method (14, 15).

Substrate—1 per cent solution of soluble starch (Noredux Standard purchased from B. Siegfried and Company, Zofingen, Switzerland) is made in a 0.02 M GP-HCl buffer, pH 6.9 (optimal for human and hog amylases) or pH 5.7 (optimal for *B. subtilis* and *A. oryzae* amylases).

Stopping Reagent—An alkaline solution of dinitrosalicylic acid is prepared as follows: 20 gm. of 3,5-dinitrosalicylic acid (lot No. 1802, Eastman Organic Chemicals Department, Division of the Eastman Kodak Company) are suspended in approximately 400 ml. of water; add dropwise and under efficient stirring a solution of 32 gm. of NaOH in 300 ml. of water, and, if necessary, gently heat on the water bath until a clear solution is obtained; then add 600 gm. of Rochelle salt in small portions and water to a final volume of 2000 ml. Filter the solution through a large coarse sintered glass filter and store at room temperature in the dark. It is stable for at least 6 months.

Assay—Add 1 ml. of the substrate solution to 1 ml. of an enzyme solution diluted in 0.002 M GP-HCl buffer at the same pH as the substrate. After exactly 3 minutes incubation at 25°, add 2.0 ml. of the stopping reagent, place the tube in boiling water for 5 minutes, cool, and dilute the reaction mixture with 20 ml. of water. The tube is read in a Klett-Summerson photoelectric colorimeter with use of the green filter No. 54. The extinction value is converted to mg. of maltose from a standard curve established with D-(+)-maltose hydrate. Best results are obtained when 1.0 ± 0.5 mg. of maltose are produced during the reaction, corresponding to the use of 0.5 to 3.0 γ of the various amylases in the assay system. When the system contains added proteases, the first dilution (1:100) is made at 0°.

Trypsin and chymotrypsin activities were measured by titrating at constant pH the hydrogen ions released during hydrolysis of benzoyl-L-arginine ethyl ester (16, 17) and acetyl-L-tyrosine ethyl ester (18), respectively. The casein digestion method of Kunitz (19) was also used.

Results

Stability of Crystalline α-Amylases in Presence of EDTA—The stability of α-amylases in the presence of EDTA varies greatly according to their origin. Whereas α-amylases from human saliva and *A. oryzae* are remarkably stable, the enzymes from hog pancreas and *B. subtilis* undergo rapid

inactivation in the presence of the chelating agent (Fig. 1). Light scattering[2] and ultracentrifugal studies indicate that the loss of activity of the latter two amylases is paralleled by a breakdown of their molecules. When, however, these two enzymes are incubated with DFP before the addition of EDTA, the breakdown is considerably retarded. As shown in Fig. 2 for the pancreatic amylase, this effect of DFP can be produced by soy bean trypsin inhibitor as well. These results indicate that inactivation of α-amylases under the above conditions is the result of a proteolytic attack, disproving earlier beliefs (6, 20) that α-amylases could be entirely freed from proteolytic activity merely by means of repeated crystallizations. The participation of proteolytic enzymes to the observed inactivations was confirmed in an experiment in which stable, DFP-treated pancreatic amylase was used. The excess DFP was removed by dialysis and the stability of this sample towards EDTA was studied in the presence of varying amounts of crystalline trypsin (as will be seen below, this α-amylase is fully stable towards trypsin in the absence of EDTA). Contamination of amylase by a fraction of a per cent of trypsin sufficed to bring about an inactivation comparable to that observed before the DFP treatment. In view of the marked difference in reactivity of soy bean trypsin inhibitor and DFP

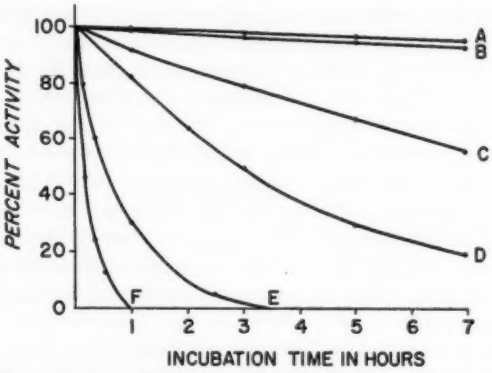

FIG. 1. Stability towards EDTA of crystalline α-amylases purified according to established procedures. 0.02 M GP buffer, 0.005 M EDTA, pH 7.2, 25°. Molar ratio, EDTA to amylase = 100:1. α-Amylases studied were as follows: Curve A, human saliva (7); Curve B, *A. oryzae* (7); Curve C, *B. subtilis*, prepared by the Enzyme Manufacturing Plant of Nagase and Company, Ltd., Amagasaki, Japan; Curve D, *B. subtilis* (7); Curve E, hog pancreas (7); Curve F, hog pancreas prepared by the Worthington Biochemical Corporation, Freehold, New Jersey, according to the method of Caldwell *et al.* (20).

[2] We are indebted to Dr. W. B. Dandliker and Dr. J. Kraut for this study.

towards trypsin and chymotrypsin (21–24), the results (Fig. 2) suggest that both proteases are present as contaminants in hog pancreas amylase. Prolonged treatment with 10^{-3} M DFP alone is apparently not sufficient to inhibit quantitatively the proteolytic activity retained by purified samples of this amylase.

Action of Metal-Binding Agents and of Proteolytic Enzymes on DFP-Treated Amylases—α-Amylases were isolated in the presence of 10^{-4} M DFP, which was added throughout the purifications and recrystallizations. The

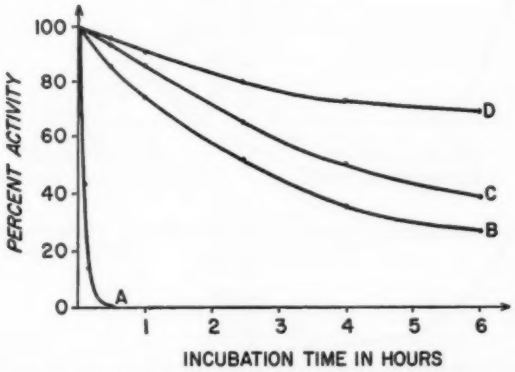

FIG. 2. Effect of protease inhibitors on the stability of hog pancreas amylase towards EDTA. The concentration of reactants was as follows: 1.6 mg. of amylase per ml.; 0.04 M phosphate buffer, pH 7.0; 10^{-2} M EDTA, 10^{-3} M DFP; 0.25 mg. of soy bean trypsin inhibitor per ml. The amylase aliquots were preincubated for 24 hours with the protease inhibitor before the addition of EDTA, and were fully stable in the absence of the metal chelate. Protease inhibitor added: Curve A, none; Curve B, soy bean trypsin inhibitor; Curve C, DFP; Curve D, soy bean trypsin inhibitor plus DFP.

stability of these freshly prepared samples was investigated in the presence of EDTA, trypsin (or chymotrypsin), and mixtures of both. EDTA was mainly used in this study, as other metal-binding agents (citrate, *o*-phenanthroline, 2,2′-bipyridine, diethyldithiocarbamate, 8-hydroxy-5-quinoline sulfonate) were found to be much less effective. It was assumed that the sole effect of EDTA on α-amylases is the binding of divalent metal ions present in the enzyme molecule (1), and that all other transformations that may take place result from this primary action.

All four amylases were found to be remarkably stable in the presence of EDTA (Fig. 3, Curves B); from pH 6.0 to 7.5, 25°, the human, bacterial, and mold amylases lost no more than 1 per cent activity per hour when incubated in a 0.5 per cent solution with 0.005 M EDTA, whereas the hog

enzyme, depending on the previous history of the sample, could lose as much as 10 per cent activity per hour (as indicated in Fig. 2, this amylase can be rendered more stable by addition of soy bean trypsin inhibitor). It should be noted, however, that the general stability range of amylases is considerably narrowed in the presence of metal-binding agents, and if the above experiments are repeated at more acidic or basic pH levels, or at higher temperatures,[3] the enzymes undergo non-enzymatic denaturation.

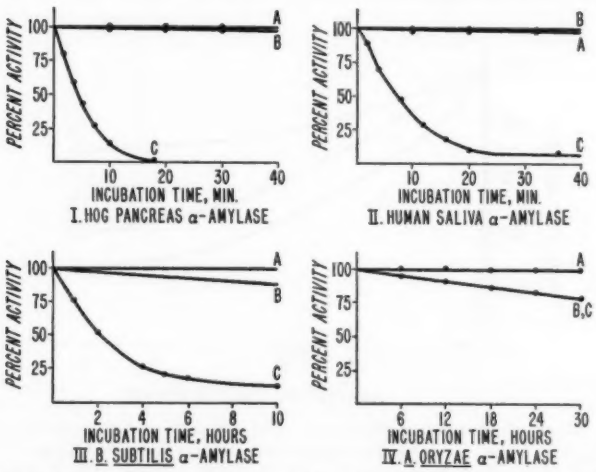

FIG. 3. Action of EDTA and proteases on α-amylases purified in the presence of DFP. The samples were incubated with trypsin (Curve A), EDTA (Curve B), and trypsin plus EDTA (Curve C), in 0.02 M GP buffer, pH 7.5, 25°. The concentration of EDTA was 0.002 M and the molar ratios, EDTA to amylase = 100:1, and trypsin to amylase = 1:1. Note that the time units in Graphs I and II are in minutes, and in Graphs III and IV, in hours.

Crystalline α-amylases are also remarkably resistant to the action of proteases. Determination of this action was, however, limited to measurements of the loss of amylase activity. Thus, only those changes affecting the structure of the active site of the enzyme are recognizable. Amylases from hog pancreas, *B. subtilis*, and *A. oryzae*[4] remain fully active when incubated for several hours at 25°, pH 7.5, in the presence of large amounts

[3] In contrast, at 3°, pH 7.0, a 0.5 per cent solution of *B. subtilis* α-amylase can be dialyzed for at least 120 hours against a continuous supply of 0.005 M EDTA, without undergoing any detectable irreversible inactivation.

[4] The resistance of mold α-amylase towards proteases was first noted 20 years ago by Akabori and Okahara (25) and led them to support the earlier opinion of Willstätter *et al.* that amylases were non-protein in nature (26).

of crystalline trypsin (Fig. 3, Curves A). Human saliva α-amylase is slightly less stable and loses approximately 1.5 per cent activity per hour under the same conditions (35 per cent within 24 hours, but none in the absence of trypsin). However, this amylase can be rendered completely resistant to the action of trypsin merely by adding Ca^{++} (10^{-2} M) to the reaction mixture.

When α-amylases are incubated with both trypsin and EDTA, a rapid loss of activity occurs in most cases, though great variations in rate may be observed from one enzyme to the other (Fig. 3, Curves C). For instance,

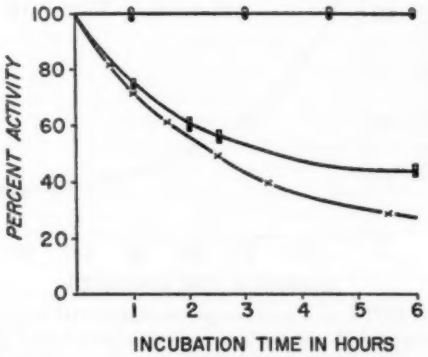

FIG. 4. Action of EDTA and proteases at 37.5° on *A. oryzae* α-amylase, purified in the presence of DFP. The sample was incubated in 0.005 M phosphate buffer, pH 7.8, in the presence of trypsin (○), chymotrypsin (●), EDTA (×); EDTA plus trypsin (□), and EDTA plus chymotrypsin (■). The concentration of EDTA was 0.003 M, the molar ratios, EDTA to amylase = 40:1, and trypsin (or chymotrypsin) to amylase = 0.2:1.

although mammalian amylases were destroyed within 0.5 hour, no further inactivation of the mold enzyme could be observed over that produced by EDTA alone. In this latter case, the experiment was repeated at a temperature (37.5°) whereby *A. oryzae* amylase undergoes non-enzymatic denaturation due to the presence of EDTA. But here again, no increase in the rate of inactivation could be observed when proteases were added to the reaction mixture (Fig. 4). The experiments involving bacteria and mold amylases (Fig. 3, Graphs III and IV and Fig. 4) were repeated, crystalline chymotrypsin being used instead of trypsin, and essentially identical results were obtained.

Mechanism of Proteolytic Degradation of α-Amylases—The above experiments clearly indicate that extensive proteolytic degradation of α-amylases occurs only through the joint action of a metal-binding agent and protease.

It seemed of interest, therefore, to investigate the mechanism of the reaction in order to obtain some information on the sequence of events leading to the breakdown of the protein molecule. To this effect, all four α-amylases were exposed to the successive action of trypsin and EDTA; characteristic results are illustrated in Figs. 5 to 7. Fig. 5 (Curves A, B, and C) shows that a preincubation of amylase with trypsin does not increase the rate at which amylase is inactivated by this protease in the presence of

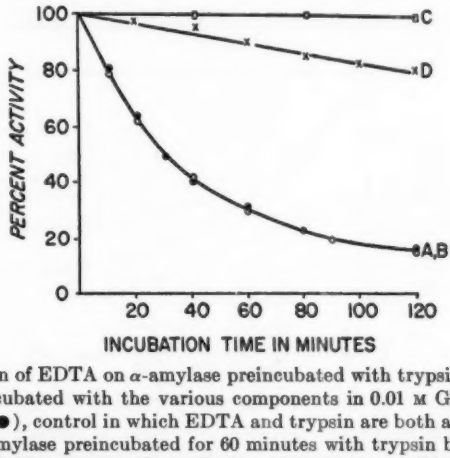

FIG. 5. Action of EDTA on α-amylase preincubated with trypsin. Hog pancreas amylase was incubated with the various components in 0.01 M GP buffer, pH 7.0, 18°. Curve A (●), control in which EDTA and trypsin are both added at zero time; Curve B (○), amylase preincubated for 60 minutes with trypsin before addition of EDTA; Curve C (□), same as Curve B, but with trypsin inhibited by addition of soy bean trypsin inhibitor 10 minutes before addition of EDTA. The concentration of EDTA was 0.002 M, the molar ratios, EDTA to amylase = 40:1, trypsin to amylase = 0.06:1, and soy bean trypsin inhibitor to trypsin = 3:1. Also illustrated is an experiment in which Ca^{++} was added to 0.002 M to the amylase solution 10 minutes before addition of EDTA and trypsin (Curve D). In this case, the concentration of EDTA was 0.004 M, to compensate for the presence of Ca^{++}.

EDTA. A control solution to which soy bean trypsin inhibitor was added after incubation with trypsin, but before the addition of EDTA, remained perfectly stable. On the other hand, when α-amylases were preincubated with EDTA before the addition of trypsin, the initial rate of inactivation upon addition of the protease was significantly increased, as can be seen by comparing Curves A and B in Fig. 6. If, on the contrary, amylase was preincubated with Ca^{++}, and then exposed to the joint action of trypsin and an excess of EDTA, the rate of inactivation was much reduced (Fig. 5, Curve D, as compared to Curve A). Here again, a control solution to which divalent metal ions were added after preincubation with EDTA, but before the addition of trypsin, remained stable (Fig. 6, Curve C); addition of the metal ions during the course of the proteolytic degrada-

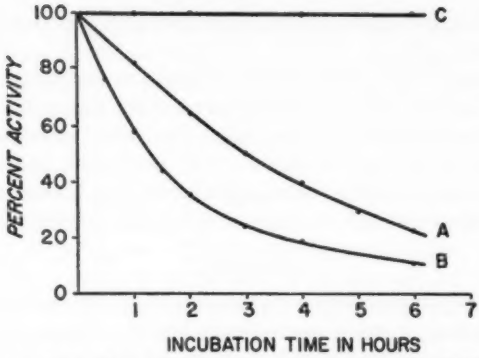

FIG. 6. Action of trypsin on α-amylase preincubated with EDTA. *B. subtilis* α-amylase was incubated in 0.02 M GP buffer, pH 7.5, 25°. Curve A, no preincubation, trypsin and EDTA both being added at zero time; Curve B, amylase preincubated for 2 hours with EDTA before addition of trypsin; Curve C, same as Curve B, but the action of EDTA is neutralized by addition of a 2-fold molar excess of either Ca^{++}, Ni^{++}, or Fe^{+++} 15 minutes before trypsin is added. The concentration of EDTA was 0.002 M, the molar ratios, EDTA to amylase = 100:1, and trypsin to amylase = 1:1.

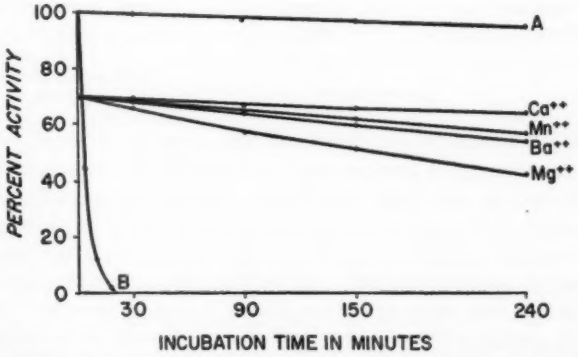

FIG. 7. Arrest of the proteolytic breakdown of amylase by divalent metal ions. Hog pancreas α-amylase was incubated in 0.02 M GP buffer, pH 7.5, 25°, in the presence of a mixture of trypsin and EDTA (Curve B). After amylase had lost 30 per cent activity (approximately 3 minutes) Mg^{++}, Ca^{++}, Ba^{++}, and Mn^{++} were added to aliquots of the reaction mixture, in a 5-fold molar excess above the concentration of EDTA. Initial concentration of EDTA was 0.002 M, molar ratio, EDTA to amylase = 100:1, and trypsin to amylase = 1:1. Also shown is a control in which the amylase was incubated with trypsin alone, without EDTA (Curve A).

tion resulted in an immediate arrest of amylase breakdown (Fig. 7). No marked specificity could be found in the protective action of divalent ca-

tions, as Mg^{++}, Ca^{++}, Ba^{++}, Mn^{++}, and Ni^{++} were all effective, although not to the same degree.

The above experiments indicate that trypsin, by itself, can neither initiate the proteolysis of native amylases, nor affect the metal-binding sites on the amylase molecule. Furthermore, they show that the removal of metal ions from the amylase molecule is fully reversible, that it constitutes a necessary preliminary step to the proteolytic breakdown of the amylases, and that it determines the over-all rate of the degradative process.

DISCUSSION

As will be shown in a subsequent publication,[5] all α-amylases investigated here contain at least 1 gm. atom of firmly bound calcium per mole. Calcium stabilizes the secondary and tertiary structures of the protein, and extensive proteolytic degradation cannot occur as long as the molecule is maintained in the proper configuration by divalent metal linkages. When, however, α-amylases are no longer combined with their full complement of metal ions, they become exceedingly susceptible to proteolysis. This is consistent with the following scheme

$$\text{Native amylase (active)} \underset{\text{Me}^{++}}{\overset{\text{metal-binding agent}}{\rightleftharpoons}} \text{"metal-poor" amylase}^{6} \text{ (active)} \xrightarrow{\text{proteases}} \text{breakdown products (inactive)}$$

The EDTA treatment, as described here, provides a sensitive test to ascertain the presence of trace amounts of protease contaminants that cannot be detected by the usual procedures. Unless special precautions are taken to inhibit or eliminate proteolytic contaminants,[7] α-amylases obtained from sources rich in proteases usually cannot tolerate such treatment without undergoing rapid destruction. Since there are no indications that precautions of this kind have ever been taken, it can be assumed that most of the work carried out on hog pancreas and *B. subtilis* α-amylases, for instance, has been performed on contaminated materials. This view is substantiated by the poor stability towards EDTA displayed by prepara-

[5] In collaboration with Dr. Bert L. Vallee, Peter Bent Brigham Hospital, Harvard University, Boston, Massachusetts.

[6] Incubation of amylase with sequestering agents under the described conditions does not necessarily remove all the bound metal ions.

[7] Short time extraction of the enzymes in the presence of non-ionic surface-active agents and purification in the presence of protease inhibitors makes possible the isolation of pure, undegraded, protease-free α-amylases (W. N. Sumerwell *et al.*, in preparation).

tions of these two enzymes purified according to various established procedures (see Fig. 1). In contrast, the atypical stability of human saliva α-amylase in the presence of EDTA, whether or not purified in the presence of protease inhibitors, is believed to result from the fact that this enzyme is the only one investigated here that is obtained from a source essentially free from proteases.[8]

Study of the mechanism of proteolytic degradation of α-amylases shows that the rate-limiting factor in the irreversible inactivation of the enzymes is the removal of the metal. This would suggest that the stability of the various α-amylases towards proteolysis is determined by the number of metal ions bound to the enzyme and the strength with which these ions are retained. These two factors vary from one amylase to the other, which explains the great differences observed in their resistance to the concerted action of trypsin and EDTA. Thus, the complete resistance of *A. oryzae* α-amylase to the action of trypsin (or chymotrypsin) in the presence of EDTA is attributed to the fact that this amylase binds its metal in such a way that it is not removed by the sequestering agent.[9] On the contrary, human saliva α-amylase loses activity in the presence of trypsin alone (no EDTA), but becomes completely resistant to this protease if calcium ions are added to the reaction mixture. The metal-binding ability of mammalian amylases appears to be much weaker than that of the other amylases investigated here; a possible explanation of this behavior pertaining to the salivary amylase might be found in the fact that this enzyme is not usually required to function in a medium rich in proteases.

The specific binding of metals by proteins is, of course, strongly dependent on the spatial configuration of the latter. Modifications of the secondary or tertiary structure of the protein might result in the easy release of metal ions otherwise very strongly held. Conditions leading to such modifications (*e.g.* variations in pH, use of organic solvent, or high salt concentrations, etc.) are often encountered during protein fractionation. Therefore, the metal content and the stability of crystalline α-amylases are certainly dependent on the previous history of the samples under investigation (*e.g.* extraction, purification, and crystallization procedures; age, storage conditions, etc.). As reported in Fig. 5 (Curve D), preincubation of a sample of crystalline hog pancreas amylase with calcium resulted in a much increased stability towards trypsin, although the protease was added together with an appropriate excess of EDTA. This

[8] Fischer and Haselbach (27) have shown that the EDTA-induced inactivation of malt α-amylase, purified (28) in the absence of DFP, is essentially reversible; this suggests that malt α-amylase is little or not associated with proteases.

[9] Recent experiments have shown that *A. oryzae* amylase retains 1 gm. atom of calcium per mole after 150 hours dialysis *versus* EDTA (to be published).

again is evidence for an enzyme partially depleted in metal and justifies the use of calcium as stabilizing agent during the purification of α-amylases.

The resistance towards proteolytic attack conferred to α-amylases by metal ions bound to their molecules explains why these enzymes can perform their physiological function in systems rich in proteases.

This work was supported by the Initiative 171 Research Fund of the State of Washington and by a research grant (No. A941) from the National Institutes of Health, United States Public Health Service.

SUMMARY

1. Hog pancreas and *Bacillus subtilis* α-amylases, purified according to the usual procedures, are still contaminated by traces of proteases that cannot be completely removed by repeated crystallizations. These contaminants catalyze the destruction of amylases when metal-binding agents are added. Pure, native α-amylases are stable in the presence of either chelating agents or proteases alone.

2. Study of the mechanism of the proteolytic degradation of α-amylases indicates that a necessary preliminary step to this process involves the release of divalent metal ions bound to the amylase molecule.

3. The instability of various α-amylases during purification and their resistance to proteolysis is discussed in the light of their respective ability to bind divalent cations.

4. Similar results obtained with α-amylases of human saliva, hog pancreas, *B. subtilis*, and *Aspergillus oryzae* suggest that the resistance towards proteolytic attack conferred to these enzymes by divalent metal ions is a general attribute of α-amylases.

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THE ENZYMATIC ESTERIFICATION OF VITAMIN A

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(Received for publication, January 29, 1958)

Vitamin A exists in two chemical forms in the body. It is found circulating in plasma as the free alcohol, but is stored in the liver primarily in the ester form. During absorption, vitamin A alcohol, either preformed or the product of hydrolysis of vitamin A esters in the lumen of the intestine, must be esterified in the intestinal wall before it can enter the circulation (2, 3). In addition to the intestinal wall, several other tissues have been reported capable of esterifying vitamin A. These include muscle (3), subcutaneous tissue (4), and kidney (5, 6).

In the eye, vitamin A is found in two tissues. The dark-adapted retina contains only traces of vitamin A, but upon exposure to light rhodopsin is bleached, liberating retinene, the aldehyde of vitamin A. Under the action of ADH,[1] retinene is rapidly converted to vitamin A alcohol. This series of reactions

$$\text{Rhodopsin} \xrightarrow{h\nu} \text{retinene} \xrightarrow[\text{DPNH}]{\text{ADH}} \text{vitamin A alcohol}$$

can be readily demonstrated in purified systems (7). However, Wald[2] has observed that part of the vitamin A formed during the bleaching of isolated retinas is esterified, indicating the presence of an esterifying system in the retina. In addition to the retina, the pigmented layers of the eye have been shown to store vitamin A, primarily as the ester (8).

We have examined the esterification of vitamin A by an enzyme system present in eye tissues, the mechanism of the reaction, and its functional significance in vision.

EXPERIMENTAL

Enzyme Preparations—Fresh cattle eyes were obtained from a local slaughterhouse. The retinas were dissected out on ice, bleached, and ly-

* This work was carried out during the tenure of a United States Public Health Service Fellowship and a National Council to Combat Blindness Fellowship. A preliminary report has appeared (1).

[1] The following abbreviations are used: ADH, alcohol dehydrogenase; DPNH, DPN, reduced and oxidized diphosphopyridine nucleotide; ATP, ADP, AMP, adenosine tri-, di- and monophosphate; CoA, coenzyme A.

[2] Wald, G., unpublished observations.

ophilized overnight. The dried retinas were ground to a fine powder, exhaustively extracted with petroleum ether, and then used directly as the enzyme source. Alternatively, the fresh retinal tissue was homogenized with 0.25 M sucrose and separated into two fractions by centrifugation at 105,000 × *g*. In addition, rod outer limbs were prepared from dark-adapted retinas by the procedure described by Wald and Brown (9).

The other tissues used as enzyme sources were the pigmented layers of the eye, which include the pigment epithelium and choroid. Owing to the fibrous nature of the choroid, the following fractionation procedure was used to isolate the enzyme. The tissues were dissected out on ice and homogenized for 2 minutes in a Waring blendor with an equal weight of finely chipped ice. After centrifugation for 10 minutes at 1800 × *g*, the supernatant fluid was decanted and the pigmented residue homogenized again for 2 minutes with additional ice. It was centrifuged for 10 minutes at 1800 × *g*, and the pooled supernatant fractions were centrifuged for 20 minutes at 35,000 × *g*. The red supernatant solution from this centrifugation was discarded, and the packed particulate material was resuspended in distilled water by homogenization in a Potter-Elvehjem homogenizer. This preparation could be stored for several months in the deep freeze with little loss of activity.

Reagents—The crystalline all-trans vitamin A alcohol used in this study was a gift of the Distillation Products Industries, a division of Eastman Kodak Company. Vitamin A acetate and palmitate were obtained from Hoffmann-La Roche, Inc. ATP, ADP, AMP, DPN, and *p*-chloromercuribenzoate were obtained from the Sigma Chemical Company, and coenzyme A from the Pabst Laboratories. Petroleum ether was Baker's Analyzed reagent, b.p. 30–60°.

Incubation Mixture—In order to add vitamin A to lyophilized enzyme preparations, it was dissolved in petroleum ether and stirred with the dry, powdered tissue. After the solvent had been removed by aspiration, the powders were suspended in neutral phosphate buffer. Several methods of adding vitamin A to aqueous enzyme preparations were tried. Solutions of vitamin A in 2 per cent digitonin or 0.1 per cent Tween 80 were effective as substrates for the enzyme, but both detergents were subsequently shown to exert an inhibitory action on the enzyme. The method finally adopted was to add vitamin A dissolved in a small volume of acetone directly to the incubation mixture. The incubations were carried out in air at 38°, with either phosphate (pH 7.0) or Veronal-acetate (pH 8.55) buffers.

Extractions—Two methods of extracting vitamin A from the incubation mixtures were used. In the first, absolute methanol was added to a final concentration of 60 per cent, the precipitated proteins were centrifuged

down, and the supernatant fluid was discarded. Anhydrous Na_2SO_4 was then added to the precipitate; the mixture ground until dry and then thoroughly extracted with petroleum ether. In the other method an equal volume of 95 per cent ethyl alcohol was added with swirling to the incubation mixture. The vitamin A could then be extracted by shaking with 2 volumes of petroleum ether, the last step being repeated twice. In either case, the pooled extracts were evaporated to dryness at reduced pressure in a warm water bath and taken up in a small volume of petroleum ether for chromatography.

Chromatography—The procedure described by Ganguly *et al.* (10) was followed for separation of the free and esterified forms of vitamin A, except that alumina (Merck aluminum oxide, suitable for chromatographic adsorption) "weakened" by the addition of 5 per cent by weight of distilled water was used.

To identify the type of vitamin A ester formed, the reversed phase chromatographic technique of Brown (11) was used. Vitamin A was located by its fluorescence under ultraviolet irradiation and by the blue color formed when the paper was sprayed with the Carr-Price reagent.

Analysis—Vitamin A was determined by the Carr-Price reaction, by using $E^{1\%}_{1\,cm.} = 4380$ at λ_{max} 618 mμ; 17 seconds after adding the antimony trichloride solution.

Results

Vitamin A in Cattle Retinas—The state of vitamin A in dark-adapted and bleached retinas is shown in Table I. In Experiment 1, retinas from dark-adapted eyes were ground with anhydrous Na_2SO_4 in dim red light, and the resulting dry powder was extracted with petroleum ether in the dark. This procedure removes the endogenous vitamin A and a small amount of free retinene, present because eyes of animals obtained at the slaughterhouse are not completely dark-adapted. Of the small amount of vitamin A present in this extract, about 60 per cent is esterified. The dry powder was then extracted with chloroform, which has been shown to bleach rhodopsin and extract the retinene which it contains (12).

When retinas are bleached by light at room temperature, most of the retinene liberated is reduced by ADH to vitamin A within 1 to 2 hours. Initially this must be the free alcohol. Yet of the vitamin A produced by bleaching retinas either directly in the excised eye (Experiment 2), or following isolation of the retina from the remaining tissue (Experiment 3), about 60 per cent is esterified.

A "cell-free" preparation of retinal tissue also can esterify vitamin A. Dark-adapted retinas are lyophilized, and any vitamin A present is removed by extracting with petroleum ether. The residue is moistened with

neutral phosphate buffer and bleached. Within the course of 1.5 hours, half of the newly formed vitamin A is found as the ester (Experiment 4). Similar results are obtained with a rod outer limb preparation from dark-adapted retinas (Experiment 5), provided that DPNH and ADH are

TABLE I

Vitamin A in Tissues and Cell-Free Preparations from Cattle Eyes

Experiment No.	Preparation	Vitamin A		Retinene	Total	Vitamin A esterified*	
		Alcohol	Ester				
		γ per eye	γ per eye	γ per eye	γ per eye	per cent	
1	Isolated retinas						
	Petroleum ether extract	0.3	0.5	0.5	1.3	62	
	$CHCl_3$ extract	0.0	0.0	5.4	5.4		
Total		0.3	0.5	5.9	6.7	62	(60–71)
2	Excised eyes, retinas bleached *in situ* 1 hr.	2.0	3.0	1.5	6.5	60	(57–60)
3	Isolated retinas bleached 1.5 hrs.	1.8	2.6	Not determined		59	(22–90)
4	Lyophilized dark-adapted retinas						
	Petroleum ether extract	0.3	1.2	0.0	1.5	80	
	Bleached 1.5 hrs.	2.3	2.2	0.0	4.5	46	
Total		2.6	3.4	0.0	6.0	57	(49–57)
5†	Lyophilized rod outer limbs						
	Petroleum ether extract	0.04	0.13	0.03		76	(68–76)
	Bleached 1.5 hrs. in the presence of DPNH and ADH	0.35	0.40	0.07		53	(53–56)
6	Pigmented layers	0.8	5.5	0.5	6.8	87	(80–93)

* The range observed in several separate experiments is given in parentheses.

† The values in Experiment 5 do not represent the actual concentration, since the preparation of rod outer limbs was not quantitative.

added during the bleaching, to facilitate the reduction of retinene to vitamin A.

The above results demonstrate the ability of preparations from retinal tissue to esterify their endogenous content of vitamin A. That such preparations are capable also of esterifying added vitamin A is shown in the following experiment. Isolated retinas were exposed to light at room temperature for 1 to 2 hours and lyophilized overnight, and the dry powder

was extracted with petroleum ether. This extraction removes all the endogenous vitamin A which had formed during and following bleaching. When vitamin A alcohol is added to this preparation, and the mixture incubated at 38° with neutral phosphate buffer, vitamin A ester is formed. The course of such an experiment is shown in Fig. 1.

Fractionation of fresh retinal tissue by homogenization in 0.25 M sucrose followed by centrifugation for 1 hour at 105,000 × *g* separates a particulate

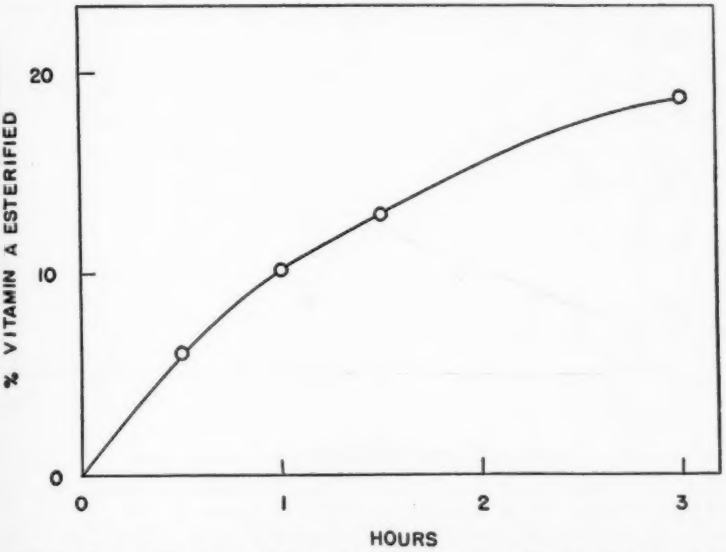

FIG. 1. The esterification of vitamin A alcohol by a lyophilized, petroleum ether-extracted powder from cattle retinas. The samples, containing 156 γ of vitamin A alcohol and the powder from 1.4 cattle retinas, were incubated at 38° in 0.067 M phosphate buffer, pH 7.0.

from a soluble supernatant fraction. These fractions, when tested alone, have only a slight activity, but when combined carry out a rapid esterification of vitamin A alcohol. The soluble supernatant fraction is heat-stable and dialyzable. As shown in Fig. 2, the amount of vitamin A ester formed is directly proportional to its concentration. The addition of ATP, CoA, cysteine, or palmitic acid had no effect on the retinal esterifying system.

When retinal homogenates are incubated for 1 hour at 38° in neutral phosphate buffer, the recovery of added vitamin A varies between 53 and 80 per cent. The recovery can be made quantitative by the addition of

α-tocopherol and the use of a N_2 atmosphere. Even when low recoveries are observed, there is no difference in the per cent esterification, indicating that those processes which destroy vitamin A apply equally to the alcohol and ester forms.

Identification of Ester Formed—The vitamin A ester formed in these isolated systems was identified by means of reversed phase paper chromatography, vitamin A acetate and vitamin A palmitate being used as standards.

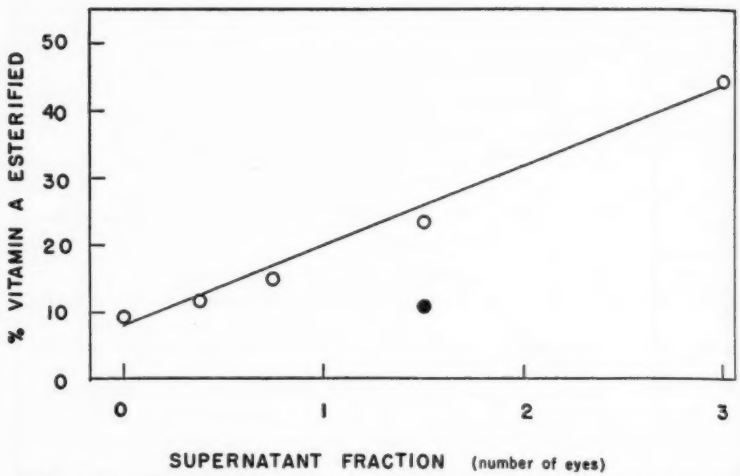

FIG. 2. Esterification of vitamin A; the effect of readding the supernatant fraction to a particulate fraction of cattle retina. Each sample contained 56 γ of vitamin A alcohol, the particulate fraction from 1.4 retinas, and 1.5 ml. of 0.067 M phosphate buffer, pH 7.0, in a final volume of 3.0 ml. The samples were incubated at 38° for 1 hour under air. The abscissa shows the amount of soluble retinal components (supernatant fraction) added in terms of the number of eyes from which it was derived. ● represents a measurement made with a sample of the supernatant fraction which had been dialyzed overnight at 4° against 0.067 M phosphate buffer, pH 7.0.

A schematic representation of a typical chromatogram is shown in Fig. 3. With use of this solvent mixture, vitamin A alcohol migrates with the solvent front. From this diagram, it appears that the vitamin A ester is formed from acids comparable in length with palmitic acid.

Pigmented Layers—The pigmented layers of cattle eyes, which include the pigment epithelium and the choroid, are an extremely active source of the esterifying enzyme. These tissues contain as much vitamin A as the retina, and, as had been previously observed (8), the majority of the vitamin is stored as the ester (Table I, Experiment 6).

The enzymatic activity is localized in the cell fraction of a water ho-

mogenate non-sedimentable at 1800 × *g*, but which forms a tightly packed residue when centrifuged at 35,000 × *g*. This particulate material is unaffected by prolonged dialysis and, unlike the retinal system, shows no increase in activity when supplemented with the supernatant fraction remaining after centrifugation at 35,000 × *g*. The rate of the reaction is optimal at pH 8.2.

Attempts were made to bring the enzyme system into solution, but without success. These attempts included extraction with strong salt solutions, treatment with deoxycholic acid or digitonin, preparation of acetone

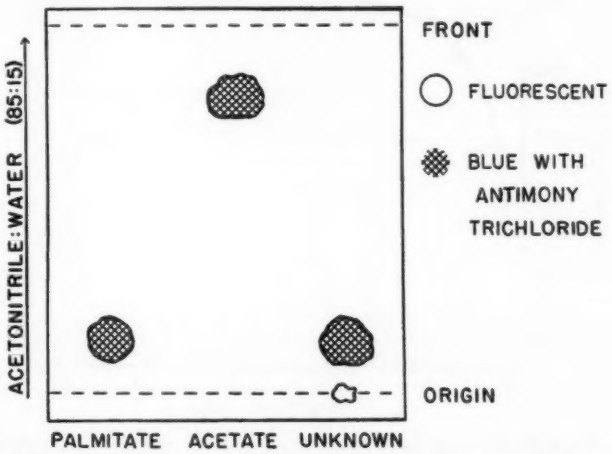

FIG. 3. Schematic representation of the reversed phase chromatography of vitamin A esters.

powders, and ultrasonic treatment. The detergents proved to be very effective inhibitors of the enzymatic activity.

When this particulate preparation is supplemented with several cofactors (ATP, palmitic acid, cysteine, and CoA), described by Kornberg and Pricer (13) for their guinea pig liver system which forms long chain fatty acid derivatives of coenzyme A, there is a marked increase in the rate of esterification of vitamin A alcohol. The effect of the individual cofactors is, however, quite different from that observed by Kornberg and Pricer (13).

Though ATP is required for the activation of long chain fatty acids in such processes as phosphatidic acid formation (14), in the present system it inhibits the esterification of vitamin A. This inhibition is observed also when the ATP is replaced by ADP or AMP. These nucleotides inhibit

the enzymatic activity 50 per cent at concentrations between 0.005 and 0.01 M.

Palmitic acid appeared to have no effect on the system when tested at 0.002 M. Other long chain fatty acids, such as oleic, stearic, myristic, and lauric acids, were tested in place of palmitic acid. These either showed no effect, or proved to be slightly inhibitory. Inasmuch as the product of this reaction is a long chain fatty acid ester of vitamin A, the lack of effect of added fatty acids is presumably due to the presence of a sufficient

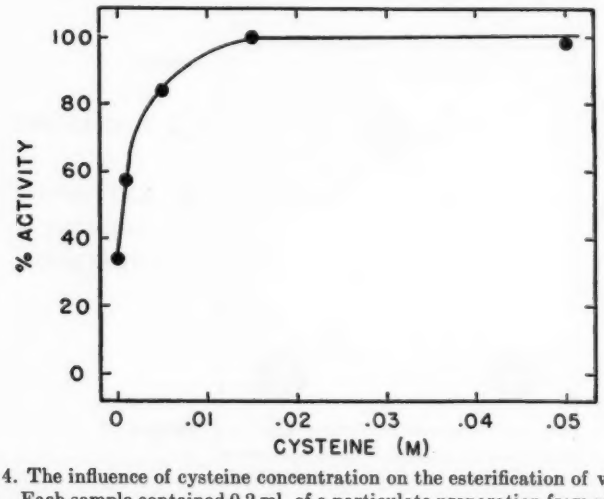

FIG. 4. The influence of cysteine concentration on the esterification of vitamin A alcohol. Each sample contained 0.2 ml. of a particulate preparation from pigmented layers of cattle eyes, equivalent to the content of one eye, 12 γ of vitamin A alcohol, 0.004 millimole Veronal-acetate buffer, pH 8.55, made up to a final volume of 4.0 ml., and incubated at 38° for 1 hour under air.

quantity of them in the enzyme preparation. This is apparently also the case in phospholipide synthesis, which can proceed in a particulate preparation without the addition of fatty acids (15).

Cysteine displays a marked effect on this system, as shown in Fig. 4. The effect, however, is not specific, for it is displayed also by reduced glutathione. This stimulation also occurs if the cysteine is added after the reaction has apparently ceased, in which case a new "burst" of esterification is observed.

Coenzyme A regularly displays a moderate influence on this reaction. In an attempt to demonstrate an absolute requirement for this factor, the tissue preparation was treated with the ion exchange resin, Dowex 1, ac-

cording to the procedure of Stadtman, Novelli, and Lipmann (16) for removing coenzyme A from cell-free preparations. This treatment resulted in only a slight loss in enzymatic activity, without any increase in the effect of added coenzyme A on the system. In the absence of added cysteine, 0.34 μmole of coenzyme A has only a negligible effect, increasing the enzymatic activity by 19 per cent. However, in the presence of cysteine, coenzyme A is active; 0.34 μmole of coenzyme A added to 100 μmoles of cysteine is as effective as doubling the cysteine concentration (enzymatic activity increases 348 per cent).

The system can be readily inhibited by the addition of small amounts of *p*-chloromercuribenzoate. This inhibition is completely abolished in the presence of cysteine. The esterification is also inhibited by NH_2OH, but in this case the addition of cysteine does not overcome the inhibition.

TABLE II

Rates of Esterification of Four Geometric Isomers of Vitamin A Alcohol

Isomer	Amount esterified	Per cent
	γ	
All-trans	4.75	(100)
Neo-b (11-cis)	4.60	97
Neo-a (13-cis)	2.85	60
Iso-a (9-cis)	2.00	42

Each sample contained 0.1 ml. of a particulate preparation of pigmented layers of cattle eyes, equivalent to one eye, 0.114 mmole of Veronal-acetate buffer, pH 8.55, 10 γ of vitamin A alcohol in 10 μliters of acetone, final volume 4.0 ml. Samples were incubated at 38° for 5 minutes under air.

Geometrical Specificity of Esterification—Vitamin A exists in the form of several well defined geometric isomers (17, 18). With respect to the regeneration of the visual pigments, it is the shape of the molecule that determines whether it reacts or not (19). The effect of the shape of the molecule on the rate of esterification has been explored. The results of one experiment are shown in Table II. Whereas the rates of esterification of all-trans and neo-b vitamin A alcohol are almost identical, the neo-a isomer is esterified at 60 per cent, and the iso-a isomer at only 42 per cent the rate of the all-trans.

Isomeric Form of Vitamin A in Pigmented Layers and Retina—The vitamin A which is present in the pigmented layers and dark-adapted retinas of cattle eyes consists of a mixture of geometric isomers. An extraordinarily large percentage of this is present as the neo-b isomer, the precursor of rhodopsin and iodopsin.

Any single isomer, or mixture of vitamin A isomers, when exposed to light in the presence of small amounts of I_2, eventually reaches an equilibrium state, containing the various isomers in fixed proportion. This equilibrium mixture or isomerate has been shown to have an $E^{1\%}_{1\,cm.}$ of 1710 in *n*-hexane (18). The results of isomerizing a tissue extract with I_2 and light are shown in Fig. 5. This is the vitamin A ester fraction from pigmented layers of cattle eyes in *n*-hexane. When the concentration of vitamin A present in this sample from a quantitative Carr-Price reaction (2.47×10^{-4} gm. per 100 ml.) is known, the $E^{1\%}_{1\,cm.}$ is found to be 1413.[3] With

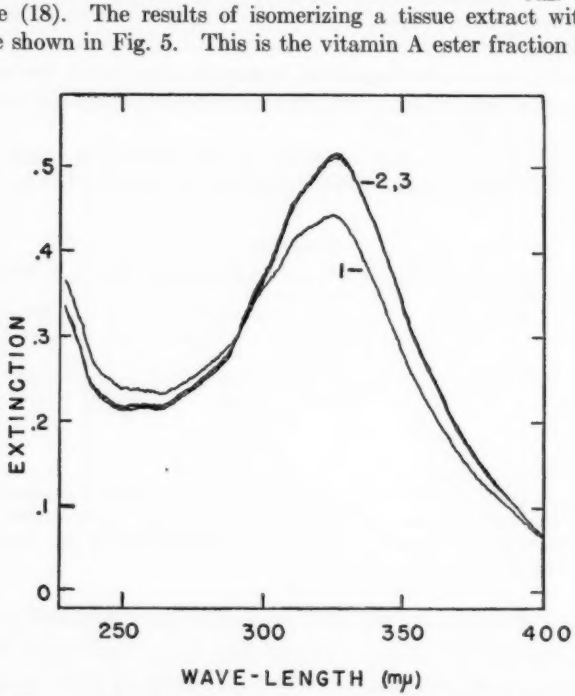

FIG. 5. Absorption spectra of a vitamin A ester fraction from pigmented layers of cattle eyes (Curve 1) before and (Curves 2, 3) after exposure to white light for 4 and 9 minutes, in the presence of 2 γ of I_2.

[3] An isomerate of this concentration should have an extinction of $(1710)(2.47 \times 10^{-4}) = 0.423$. The difference between this value and the one actually observed (0.516) is due to extraneous absorption in this region of the spectrum by other components present in the tissue extract. Subtracting this difference (0.093) from the extinction of the solution before isomerization, and dividing by the concentration, gives the $E^{1\%}_{1\,cm.}$ of the initial solution. This then is $(0.442 - 0.093)/(2.47 \times 10^{-4}) = 1413$.

use of this figure, the fraction of neo-b in the vitamin A ester of cattle pigmented layers is found to be 65 per cent.[4] Similarly high proportions of neo-b are found also in the alcohol fraction from pigmented layers and in the extracts of dark-adapted retinas.

Vitamin A Esterase—The fact that both the alcohol and ester fractions of pigmented layers of cattle eyes contain high proportions of neo-b vitamin A would seem to indicate that an equilibrium exists between the free and esterified forms of vitamin A in this tissue. We have been unable, however, to demonstrate clearly the hydrolysis of long chain fatty acid esters of vitamin A, either natural or synthetic (palmitate), when added to homogenates obtained from the pigmented layers. On the other hand, lyophilized retinal preparations are capable of hydrolyzing vitamin A esters. The esters tested included the acetate and the palmitate, the former being hydrolyzed much more rapidly than the latter.

TABLE III

Distribution of Vitamin A Esterifying Enzyme in Cattle Pigment Epithelium and Choroid

Tissue	Dry weight per eye	Enzyme distribution		
	mg.	*units* per mg. dry weight*	*total units*	*per cent*
Pigment epithelium	4.3	10.41	44.8	72
Choroid	116.7	0.148	17.3	28

* A unit of enzyme is defined as the amount which catalyzes the formation of 1 mμmole of vitamin A ester when incubated at 37.5° for 30 minutes, in a solution containing 77 mμmoles of vitamin A alcohol and cysteine (0.033 M), pH 8.55.

Distribution of Esterifying Enzyme in Pigmented Layers—By taking advantage of the fact that the pigment epithelium is a tissue with only a single cell layer, it has been possible to separate it from the underlying

[4] Of the five known isomers of vitamin A, iso-a and iso-b vitamin A do not appear to be present in extracts of pigmented tissues of cattle, inasmuch as these extracts, when tested for their ability to regenerate visual pigments, form only rhodopsin, without admixture of isorhodopsin, the photosensitive pigment formed from either of these isomers (19). Thus the vitamin A from pigmented layers is made up of a mixture of all-trans, neo-a, and neo-b vitamin A. If we assume that the first two are present in the proportion 85/15, as has been found in rat liver (20), their mixed $E^{1\%}_{1\text{ cm.}}$ would be 0.85(1820) + 0.15(1690) = 1800. The observed value, 1413, would be composed of contributions from neo-b, $E^{1\%}_{1\text{ cm.}} = 1200$, and the all-trans neo-a mixture, $E^{1\%}_{1\text{ cm.}} = 1800$. If we let x = fraction of vitamin A present as neo-b, $1 - x$ = fraction of vitamin A present as all-trans + neo-a, then $x(1200) + (1 - x)(1800) = 1413$ and $x = 65$ per cent. The $E^{1\%}_{1\text{ cm.}}$ values are taken from the paper of Brown and Wald (18).

choroid.[5] After removing the retina, the eye cup is gently rinsed with a few ml. of saline to remove adhering rod outer limbs. The majority of the pigment epithelium is then removed by rubbing a fine camel hair brush over the surface of the eye cup. The remaining pigment epithelium and choroid can then be teased away from the sclera. By using this procedure, over 70 per cent of the enzyme is found in the pigment epithelium, as seen in Table III. Since the recovery of the pigment epithelium by this procedure is far from complete, this measurement makes it probable that virtually all esterifying activity is in the pigment epithelium and little if any in the choroid.

Distribution of Esterifying Enzyme in Other Tissues—In addition to cattle retinas and pigmented layers, various other tissues were tested for their ability to esterify vitamin A alcohol. Rat kidney, liver, and small intestine all failed to show significant rates of esterification.

DISCUSSION

The demonstration of enzyme capable of esterifying and hydrolyzing vitamin A in the tissues of the eye does not necessarily mean that a simple equilibrium exists between the vitamin A of the eye and the rest of the body. Vitamin A is transported in the blood as the free alcohol, whereas it is found deposited in the liver, the main storage depot of vitamin A in the body, primarily as the ester (3). There does not appear to be any direct relationship between liver and blood vitamin A, for the latter tends to remain at a fairly constant level, despite wide variations in the liver level, until such time as the liver stores are depleted, when the blood level falls (21). It is of interest that vitamin A is deposited also in the pigmented layers and retina primarily as the ester. Vitamin A appears to be held tenaciously by the eye tissues as compared to the liver.[6] This has been demonstrated by Brenner *et al.* (22) who studied vitamin A depletion in either young rats or those made hypervitaminotic with respect to vitamin A. They showed that the eye is capable of holding on to vitamin A even after the liver stores have been completely depleted, and the blood level had fallen to half of its original value. This was also shown histologically by Greenberg and Popper (23), who found that the vitamin A

[5] We are indebted to Dr. A. M. Potts, Western Reserve University, for describing this method.

[6] This has been recently discussed by J. E. Dowling and G. Wald (*Proc. Nat. Acad. Sc.*, in press). They found that rats, maintained on a vitamin A-free diet, first lost all of their liver stores of vitamin A and then the blood vitamin A level fell rapidly to zero from its normal level. It was only at the time that vitamin A was no longer detectable in the blood that retinal vitamin A, bound in the form of rhodopsin, began decreasing.

fluorescence of the retina and pigment epithelium of rat eyes was almost independent of the amount of vitamin A stored in the liver and was only slightly decreased when vitamin A was no longer detectable in the liver. The ciliary process of the eye also displayed vitamin A fluorescence, but this fluorescence disappeared in vitamin A-deficient animals, indicating its dependence on the vitamin A nutritional status.

Further evidence that the vitamin A stored in the eye tissues is not in complete equilibrium with the vitamin A stored in such organs as the liver is shown in a study by Wald (24) on the distribution of vitamins A_1 and A_2 in the eye tissues and liver of various marine and fresh water fishes. Many of these contain a mixture of vitamin A_1 and A_2 in the eye and liver tissues, and, in several cases, the ratios of vitamin A_1 to vitamin A_2 in these tissues are reversed. Whenever a marked difference exists between the vitamin A_1 to vitamin A_2 ratio in the retinas and the livers, the vitamin A distribution in the pigmented layers parallels the retinal ratio, but not the liver ratio.

Of greatest importance with respect to the visual system is the difference in distribution of vitamin A isomers in the eye tissues from that in the rest of the body. As we have shown, a large percentage of the vitamin A present in pigmented layers of cattle eyes and retina is the neo-b isomer, the precursor of rhodopsin. A recent study, in which the sensitive assay for ability to form rhodopsin was used, has failed to detect this isomer in either cattle plasma or liver.[7] This fact, coupled with the retention by the eye of vitamin A during depletion, points to a mechanism for isolating the vitamin A metabolism of the eye from the rest of the body.

This mechanism may be associated with the rapid and relatively selective esterification of neo-b and all-trans vitamin A in the pigment epithelium. In this way, the active isomer for rhodopsin regeneration, neo-b vitamin A, which is formed only in the eye (25), and its immediate precursor, all-trans vitamin A, would tend to be concentrated at the expense of the other isomers. The esters would then become available to the retina, presumably by diffusion across the wide surfaces of contact between the pigment epithelium and rods and cones. It is interesting that the retina alone among the eye tissues readily hydrolyzes vitamin A ester, thus making it available for oxidation by the alcohol dehydrogenase of the retina and rhodopsin formation.

SUMMARY

A cell-free enzyme preparation capable of esterifying vitamin A alcohol has been obtained from various eye tissues. The enzyme is concentrated in the pigment epithelium, where it is found in a particulate fraction. The

[7] Wald, G., and Brown, P. H. S., unpublished observations.

pH optimum for the reaction is at 8.2, and the activity is greatly enhanced by the addition of sulfhydryl compounds. The product of the reaction is a long chain fatty acid ester of vitamin A. Of the eye tissues studied, only the retina is capable of hydrolyzing long chain fatty acid esters of vitamin A.

The pigment epithelium enzyme displays relative geometric specificity towards the various isomers of vitamin A. This may account for the high proportion of neo-b vitamin A stored in the eye.

The author wishes to express his appreciation to Dr. George Wald for many helpful discussions during the course of this work and particularly during the preparation of this manuscript.

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SYNTHESIS OF SATURATED AND UNSATURATED PHOSPHATIDYL GLYCEROLS

III. CARDIOLIPIN SUBSTITUTES*

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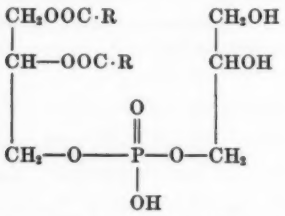
(Received for publication, January 30, 1958)

Recently, Benson and Maruo,[1] separating the phospholipides of an ethanolic extract of the alga *Scenedesmus* by chromatographic means, obtained a fraction whose major constituent was identified by its reaction with lead tetraacetate, formation of benzoyl- and isopropylidene derivatives, and liberation of α,α-diglycerophosphate on mild alkaline hydrolysis as an α-phosphatidyl-α-glycerol (Fig. 1, Structure I). Structural details remaining to be determined are the nature of the fatty acid substituents and, if these are not identical, their relative positions on the substituted glycerol moiety, as well as the configuration of both glycerol moieties. Theoretical considerations predict the existence of four stereoisomers grouped in two pairs of enantiomers for an α-phosphatidyl-α-glycerol containing two identical fatty acid substituents (Fig. 1, Structures II to V). Several methods of determining the structure and configuration of naturally occurring phosphatidyl glycerols can be envisaged. However, the following two procedures seem to be the most promising ones. One requires the removal of the fatty acid substituents by a mild alkaline hydrolysis and comparison of the saponification product with bisglyceryl phosphates of known structure and configuration, while the other involves the synthesis of phosphatidyl glycerols of known structure and configuration for comparison with the natural product. However, Structures III and IV would be distinguished only by the second method. A method for the synthesis of bis(L-α-glyceryl)phosphoric acid and bis(D-α-glyceryl)-phosphoric acid has been reported recently by the present authors (3). The synthesis of α-phosphatidyl-α-glycerols has now been effected in this laboratory. As we pointed out earlier (3), there are good reasons for expecting the naturally occurring phosphatidyl glycerols to be derivatives of bis(L-α-glyceryl)phosphoric acid. Hence we concerned ourselves first with the synthesis of L-α-phosphatidyl-L-α-glycerols. In the absence of any information as to the nature of the fatty acid substituents of the natural

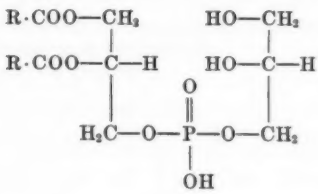
* The synthesis of enantiomeric α-phosphatidic acids (1) and α-bisphosphatidic acids (2) should be considered Papers I and II in this series.

[1] Benson and Maruo (14).

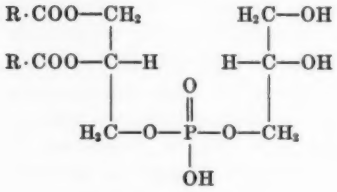
products, both an unsaturated and a saturated phosphatidyl glycerol were prepared containing oleic acid and stearic acid, respectively. The synthesis of (dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol from D-mannitol involves the following nine intermediates: 1,2,5,6-diacetone D-mannitol → acetone D-glyceraldehyde (4) → D-acetone glycerol (4) → D-acetone glycerol



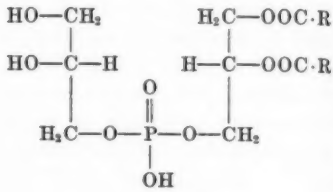
I. Structure of phosphatidyl glycerol as suggested by Benson and Maruo's evidence.



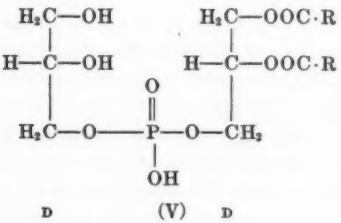
L (II) L



L (III) D



L (IV) D

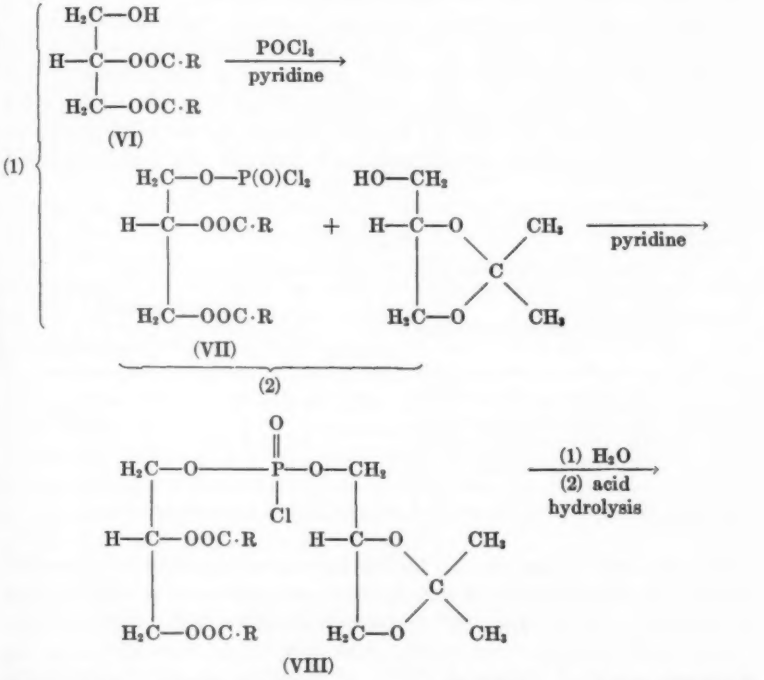


D (V) D

FIG. 1. Phosphatidyl glycerol and its possible stereoisomers

benzyl ether (5, 6) → L-α-glycerol benzyl ether (5, 6) → D-α,β-(bis-9,10-dibromo)distearin benzyl ether (7) → D-α,β-(bis-9,10-dibromo)distearin (7) → D-α,β-diolein (7) → isopropylidene(dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol. The details of the last steps of the synthesis are shown in Scheme 1. The isopropylidene(dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol was not isolated, since at some stage during the working up and the addition of water to decompose the phosphorochloridate (VIII) cleavage of

the acetone group takes place, yielding directly the dioleoyl phosphatidyl glycerol (II). The unsaturated phosphatidyl glycerol (II) was obtained as a viscous oil in an over-all yield of 90 per cent calculated from diolein. In selecting for the synthesis of dioleoyl phosphatidyl glycerol (II), D-α,β-



(Dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol

(Distearoyl-L-α-glycerylphosphoryl)-L-α-glycerol

R = $-(CH_2)_7CH{=}CH(CH_2)_7CH_3$,
R_1 = $-(CH_2)_{16}CH_3$

SCHEME 1. Synthesis of α-phosphatidyl-α-glycerols

diolein and D-acetone glycerol, whose primary hydroxyl groups are derived by reduction of the carbonyl group of D-glyceraldehyde, the phosphoric acid residue is placed in a position on both glycerol moieties that is opposite to that of the phosphoric acid radical in D-glyceraldehyde-3-phosphoric acid and its reduction product D-α-glycerolphosphoric acid. Thus both glycerol moieties of the phosphatidyl glycerols II and IX have spatial arrangements opposite to those of D-glyceraldehyde-3-phosphoric acid and D-α-glycerolphosphoric acid and hence possess the L configuration. The enantiomer of (dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol and the other pair of enantiomers (III and IV) may be obtained by the same procedure by using the appropriate stereoisomers of α,β-diolein and acetone glycerol (Table I).

TABLE I

Synthesis of Diastereoisomers of α-Phosphatidyl-α-glycerols

Desired (dioleoyl)phosphatidyl glycerol	Required stereoisomers of	
	α,β-Diolein	Acetone glycerol
II. (DO-L-α-G)(L-α-G)PA	D	D
III. (DO-L-α-G)(D-α-G)PA	"	L (8)
IV. (DO-D-α-G)(L-α-G)PA	L (7)	D
V. (DO-D-α-G)(D-α-G)PA	"	L

Abbreviations, DO = dioleoyl, G = glyceryl, PA = phosphoric acid.

The catalytic reduction of (dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol (II) in ethanolic solution with hydrogen and platinum as catalyst gave an excellent yield of (distearoyl-L-α-glycerylphosphoryl)-L-α-glycerol (IX).

This work completes the synthesis of a series of naturally occurring phosphate esters of increasing degree of substitution: glycerophosphoric acid (9, 10), bisglycerylphosphoric acids (3), phosphatidic acids (1), phosphatidyl glycerols, and bisphosphatidic acids (2). Members of these classes are now accessible as individual, stereochemically pure compounds as a result of syntheses developed in this laboratory. These methods permit the preparation of unsaturated, as well as saturated, representatives of the acylated glycerol phosphates. The synthesis of an unsaturated phosphatidic acid and bisphosphatidic acid will be reported soon.

Both the saturated and unsaturated phosphatidyl glycerols, which are closely related in structure to cardiolipin (11), are under investigation by Dr. R. H. Allen and Dr. D. B. Tonks in the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, as possible substitutes for cardiolipin in the serodiagnosis of syphilis. This work will be reported

independently elsewhere. Preliminary indications are that the unsaturated phosphatidyl glycerol is fairly reactive as a cardiolipin substitute in VDRL tests.

EXPERIMENTAL

(Dioleoyl-L-α-Glycerylphosphoryl)-L-α-Glycerol (II)—In a dry 300 ml. 3-necked round flask with ground glass joints equipped with an oil-sealed motor-driven stirrer, calcium chloride tube, and dropping funnel were placed 3.1 gm. (0.02 mole) of freshly fractionated phosphorus oxychloride. The flask was immersed in an ice bath, and a mixture of 12.42 gm. (0.02 mole) of D-α,β-diolein (7) and 1.58 gm. (0.02 mole) of anhydrous pyridine was added dropwise with stirring to the phosphorus oxychloride during 15 minutes. The funnel was rinsed with a solution of 1.6 ml. of pyridine in 10. ml of anhydrous ether, which was added to the reaction mixture, and the stirring was continued, the mixture being kept for 1 hour at 0° and 1 hour at room temperature (approximately 24°). At the end of this period, the flask was placed in a water bath at 10°, and a solution of 2.65 gm. (0.02 mole) of D-acetone glycerol (4) and 1.60 ml. of pyridine in 20 ml. of ethanol-free and anhydrous chloroform was added over a period of 5 minutes. The mixture was brought to room temperature, and the stirring was continued for 2½ hours, 0.36 ml. (0.02 mole) of distilled water being added at the end of the 2nd hour. The reaction mixture was diluted with 300 ml. of ether, the pyridine hydrochloride was removed by filtration, and the filtrate was washed in succession with three 200 ml. portions of ice-cold 2 N sulfuric acid, two 200 ml. portions of a saturated sodium bicarbonate solution, and two 200 ml. portions of water. The ether solution was dried with anhydrous sodium sulfate, the solvent was distilled off under reduced pressure, and the residue was kept in a high vacuum at 35–40° until its weight was constant. The product (15.5 gm., P = 3.85) was dissolved in 200 ml. of anhydrous acetone; the solution was cleared by centrifugation and kept for 2 hours at −85° in a bath of solid carbon dioxide and acetone. The mixture was centrifuged in a refrigerated centrifuge at as low a temperature as possible, the supernatant solution was decanted, and the precipitate was kept *in vacuo* until its weight was constant. The residue, a viscous oil, was free of chemically bound acetone, weighed 14.0 gm. (90.3 per cent of the theoretical amount), and consisted of pure (dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol; n_D^{23} 1.4705, $[\alpha]_D^{21}$ +2.0° in chloroform (c = 10), $[\alpha]_D^{22}$ +2.35° in 99 per cent ethanol (c = 11). On titration in aqueous emulsion, by the method of Fleury, the compound consumed per mole, 1.02 moles of periodic acid. It was found to be highly soluble at room temperature in methanol, ethanol, acetone, chloroform, glycol mono-

methyl ether, diethyl ether, petroleum ether, or benzene, but insoluble in water, with which it readily forms a stable emulsion.

$C_{42}H_{79}O_{10}P$ (775).	Calculated.	C 65.07, H 10.27, P 4.00, iodine No. 65.5
	Found.	" 64.86, " 10.20, " 4.03, " " 64.0

Recovery of Oleic Acid—A solution of 289.0 mg. of (dioleoyl-L-α-glyceryl-phosphoryl)-L-α-glycerol in a mixture of 25 ml. of ethanol and 25 ml. of a 2 N aqueous solution of potassium hydroxide was boiled under reflux for 6 hours. The solution was then concentrated to approximately one-half of its volume by distillation under reduced pressure, and the concentrate was acidified with 7 ml. of 10 N sulfuric acid. The oleic acid was extracted with four 25 ml. portions of petroleum ether (b.p. 30–60°), the combined extracts were evaporated in a stream of nitrogen at a bath temperature of 50–60°, and the oleic acid was determined by alkali titration with thymol blue as indicator (12). Oleic acid calculated, 210.6 mg.; found, 210.3 mg. (99.8 per cent of theory).

(*Distearoyl-L-α-Glycerylphosphoryl*)-*L-α-Glycerol* (*IX*)—A solution of 4.0 gm. of (dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol in 60 ml. of 99 per cent ethanol was placed in an all-glass reduction vessel, 0.4 gm. of platinic oxide (Adams' catalyst[2]) was added, and the mixture was shaken in an atmosphere of pure hydrogen[3] under an initial pressure of approximately 50 cm. of water until the consumption of hydrogen ceased, about 20 minutes being required. The apparatus was flushed with nitrogen, the saturated phosphatidyl glycerol was brought into solution by placing the reaction vessel in a bath of warm water, and the catalyst was removed by centrifuging the warm solution. The catalyst was washed twice with small portions of warm ethanol, and the combined ethanolic solutions were placed in an ice box to crystallize. The mixture was filtered with suction on a Buchner funnel, and the solid material, weighing 3.9 gm. (97 per cent of theory), was recrystallized from 40 ml. of anhydrous acetone, giving 3.7 gm. (92.0 per cent of theory) of (distearoyl-L-α-glycerylphosphoryl)-L-α-glycerol (IX); m.p. 66.5–67.0°, $[\alpha]_D^{22}$ +2.0° in chloroform (c = 10). The substance, which was free of potassium,[4] was found to be highly soluble at room temperature in chloroform or benzene and in warm methanol, ethanol, acetone, glycol monomethyl ether, ether, or petroleum ether.

$C_{42}H_{83}O_{10}P$ (779).	Calculated.	C 64.75, H 10.74, P 3.97
	Found.	" 64.73, " 10.81, " 3.90

[2] The catalyst was prepared as described by Adams, Voorhees, and Shriner (13), except that the sodium nitrate was replaced by an equimolecular amount of potassium nitrate.

[3] Electrolytically produced hydrogen was used.

[4] A possible contamination introduced by Adams' catalyst.

SUMMARY

A procedure has been developed for the synthesis of α-phosphatidyl-α-glycerols, permitting the preparation of all four stereoisomers. The synthesis of (dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol from D-α,β-diolein and D-acetone glycerol via isopropylidene(dioleoyl-L-α-glyceryl-phosphoryl)-L-α-glycerol, and the catalytic reduction of the unsaturated phosphatidyl glycerol to (distearoyl-L-α-glycerylphosphoryl)-L-α-glycerol are described in detail.

This work was assisted with funds allocated by the Province of Ontario under the National Health Grants Program of the Department of National Health and Welfare, Ottawa.

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THE CHEMISTRY OF THE NATIVE CONSTITUENTS OF THE ACETONE-SOLUBLE FAT OF MYCOBACTERIUM TUBERCULOSIS (BREVANNES)*

I. GLYCERIDES AND PHOSPHOGLYCOLIPIDES

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(Received for publication, January 21, 1958)

In the course of their pioneering studies on the chemical composition of the lipides of *Mycobacterium tuberculosis*, Anderson and his coworkers developed a general extraction procedure (1). According to their scheme, the cultures are exhaustively extracted with a mixture of ethanol-ether (1:1) and then with chloroform. By further solvent fractionation the ethanol-ether-soluble lipides are then separated into the "acetone-soluble fat" and the acetone-insoluble "crude phosphatide," while the chloroform extracts are divided into the methanol-soluble "soft wax" and the methanol-insoluble "purified wax." None of these fractions, however, is chemically homogeneous.

The discovery of the cord factor by Bloch (2) stimulated new interest in the chemistry of these fractions and led to a search for better methods of purification. Progress toward the isolation of the pure native lipides was made by the introduction of chromatography (3–6) and infrared spectroscopy (5, 7). The application of these methods resulted in the isolation and subsequent elucidation of the chemical structure of the toxic cord factor (8–11) and of several other native constituents of Anderson's purified wax (12, 13).

The present investigation extends our previous work on the composition of the chloroform extracts (12) to include the acetone-soluble fat fraction of the methanol-ether extracts. In contrast to these previous studies, the present paper and Paper II, however, deal exclusively with fractions obtained from the virulent human strain Brévannes. This qualification seems indicated since Anderson, who in his careful and comprehensive work has laid the ground for all subsequent studies in this field, already emphasized the dependence of the lipide composition upon the nature of the

* This investigation was supported by research grants No. E-1500 and No. E-1297 from the Divsion of Research Grants of the National Institutes of Health, Public Health Service.

A portion of this work was carried out at the Public Health Research Institute of the City of New York, Inc., New York, New York.

strains and the cultural conditions (14). These observations were confirmed in later investigations, notably by Lederer and coworkers (15), by Kubica *et al.* (16), and in our studies on the native constituents of the Wax C fraction (12).

With use of the strain Brévannes and by applying chromatographic methods, Aebi *et al.* initiated the chemical study of the native constituents of this fraction (6). The present two reports give additional information obtained mainly by extensive infrared analysis of the chromatographically purified components. The first paper deals with the fractionation procedures and the characterization of some glyceride and phosphoglycolipide fractions, while the subsequent report describes a new crystalline naphthoquinone related to vitamin K_2.

EXPERIMENTAL

Isolation of Acetone-Soluble Fat

Cultural and extraction procedures have been described in a previous paper (8). 3 to 4 week-old surface cultures of a virulent human strain of *M. tuberculosis* (Brévannes) were used. The methanol-ether (2:1) extracts from various batches were concentrated *in vacuo*; the residue was dark brown and salve-like. The acetone-soluble fat was prepared by solvent fractionation according to Aebi *et al.* (6) and had the appearance of a brown viscous oil.

Chromatographic Partitioning

Further partitioning of the acetone-soluble fat was effected by chromatography on magnesium silicate-Celite (17) and silicic acid (Mallinckrodt) columns. The preparation of the magnesium silicate-Celite and the chromatographic procedures have been described (5). Fractions[1] eluted from the columns were examined by infrared spectrophotometry (Tables I and II).

From well established correlations the main components can be classified according to their infrared spectra[2] as belonging to one of the following groups: I, glycerides; II, phosphoglycolipides; III, naphthoquinone derivatives.

[1] Chromatographic fractions are referred to by the roman numerals used for the chromatograms represented in Tables I to III, followed by the letters designating the corresponding fractions.

[2] Some of the infrared spectra were recorded with a Perkin-Elmer model No. 21 double beam infrared spectrophotometer at the American Cyanamid Company, Stamford, Connecticut; the others were taken with a Beckman model IR-4 double beam instrument at the Department of Microbiology, School of Medicine, University of Pittsburgh. The authors are greatly indebted to Dr. R. C. Gore and Mr. N. B. Colthup, American Cyanamid Company, for generous assistance and helpful discussions.

TABLE I

Chromatogram I. Chromatography of Acetone-Soluble Fat Brévannes on Silicic Acid

Fraction	Eluted with	Aspect of material	Characteristic infrared* absorptions	Interpretation
a	Petroleum ether-benzene, 4:1	Light yellow oil	1740 (s.), 1665 (m.), 1600 (w.), 1175 (m.), 720 (m.)	Mixture of triglyceride and naphthoquinone derivative
b	Petroleum ether-benzene, 1:1	Light orange oil	1740 (w.), 1665 (s.), 1625 (m.), 1600 (s.), 1450 (s.), 1385 (s.), 1330 (s.), 1300 (s.), 720 (s.)	Naphthoquinone derivative + small amount of triglyceride
c	Petroleum ether-benzene, 1:1	Light yellow wax	1740 (s.), 1470 (s.), 1235 (m.), 1160 (s.), 1110 (m.), 720 (m.)	Triglyceride
d	Benzene-ether, 1:1	Red-brown soft solid	3500 (m.), 1750–1700 (s.), 1470 (s.), 1250 (m.), 1160 (s.), 1110 (m.), 720 (m.)	Mono- and diglycerides (Smith's Compound C)
e	Benzene-ether, 1:1	Brown viscous oil	3450 (s.), otherwise similar to Fraction Id	Somewhat more monoglycerides
f	Ether-methanol, 19:1	Dark brown solid	See Chromatogram II, Fraction d	Smith's Compound D
g	Ether-methanol, 19:1	Dark brown gum	3350 (s.), 1715 (m.), 1660 (s.), 1620 (m.), 1600 (m.), 1465 (s.), 1380 (s.), 1335 (m.), 1295 (s.), 1260 (m.), 1220 (m.), 1180 (s.), 1060 (m.), 975 (m.), 915 (m.), 835 (w.), 790 (w.), 720 (m.)	Mixture of monoglycerides and naphthoquinone derivative
h	Ether-methanol, 19:1	Dark brown gum	Same absorptions as Fraction Ig but weaker C=O band (1715)	Naphthoquinone derivative; some monoglycerides
i	Ether-methanol, 19:1	Dark brown viscous fluid	Same absorptions as in Fractions Ig and Ih, additional strong bands at 3350, 1110, 1040, 990, 915	Same components as in Fraction Ih + free glycerol
k	Ether-methanol, 19:1	Light brown oil	3350 (s.), 1650 (broad), broad overlapping bands 1500–1200, 1110 (s.), 1040 (s.), 995 (m.), 925 (m.), 860 (m.)	Mostly free glycerol

* Estimated relative intensity of absorption bands is expressed by the symbols s. = strong, m. = medium, w. = weak.

Group I. Glycerides

That the lipides of *M. tuberculosis* are particularly rich in mono-, di-, and triglycerides of various long chain fatty acids has been shown in studies on the native constituents of the Wax C fraction of Anderson's purified wax (12) and Dubos' "toxic lipide" (13).

Smith and coworkers isolated from ethanol-ether extracts of a large number of mycobacterial strains compounds designated A, B, C, D, and E,

TABLE II

Chromatogram II. Chromatography of Acetone-Soluble Fat Brévannes on Magnesium Silicate-Celite

Fraction	Eluted with	Aspect of material	Characteristic infrared* absorptions	Interpretation
a	Petroleum ether	Dark brown oil		Complex mixture
b	Petroleum ether	Light yellow wax	3350 (m.), 1730–1710 (s.), 1475 (s.), 1425 (m.), 1395, 1385 (m.), 1335–1240 succession of 7 closely spaced sharp bands (m.), 1223, 1205, 1185 (s.), 1140 (m.), 1105, 1075 (w.), 945 (m.), 875 (w.), 720 (m.)	Smith's Compound C
c	Petroleum ether-benzene, 4:1	Light brown wax	Same as Fraction IIb	Smith's Compound C
d	Petroleum ether-benzene, 1:1	Softred wax	3350 (shoulder), 3250 (s.), 1725 (s.), 1475 (s.), 1425, 1395, 1380 (m.), 1330–1240 succession of closely spaced not completely resolved bands (m.), 1222 (s.), 1200 (s.), 1182 (s.), 1140, 1120, 1105, 1060 (m.), 1045 (s.), 990, 940 (m.), 880–820 3 weak bands, 720 (m.)	Smith's Compound D
e	Benzene-ether, 1:1	Brown gum	3350 (s.), 1740–1720 (s.), 1660, 1620 (m.), 1600 (w.), 1470 (s.), 1380 (m.), 1350–970 band with 3 broad maxima near 1250, 1110, 1060 (s.), 940 (w.), 860 (m.) (broad), 720 (w.)	Phosphoglycolipides; some naphthoquinone derivatives
f	Ether-methanol, 9:1	Yellowish oil	Same absorptions as Fraction Ik	Glycerol

* Estimated relative intensity of absorption bands is expressed by the symbols s. = strong, m. = medium, w. = weak.

which were characterized by infrared spectroscopy (16) and more recently reported to be glycerides (18).

Spectral correlations (12, 19–22) showed that Fraction Ic which resembled Smith's Compound A consisted of triglycerides. Fractions If and IId, which were spectroscopically identical with Smith's Compound D (Fig. 1, Spectrum 3), behaved chromatographically[3] as a monoglyceride.

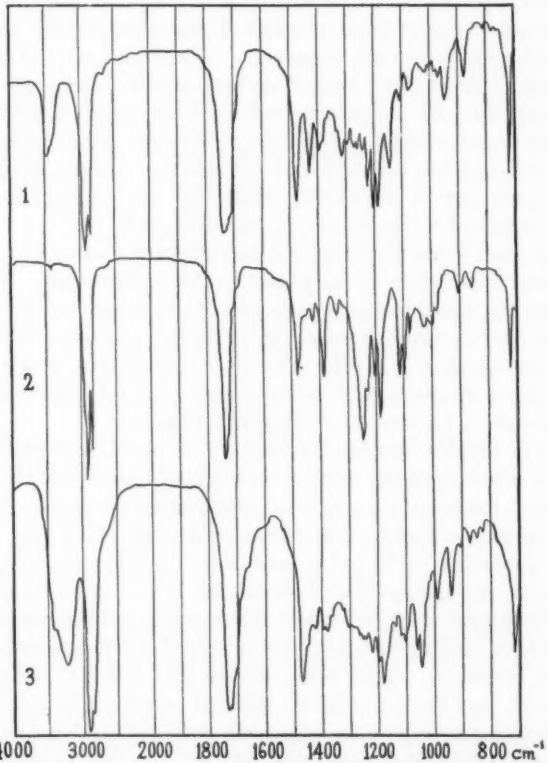

FIG. 1. Infrared spectra of glyceride fractions (taken from melts). Spectrum 1, Smith's Compound C (diglyceride); Spectrum 2, acetylated Compound C; Spectrum 3, Smith's Compound D (monoglyceride).

Furthermore, the infrared spectrum of Compound D is similar to the spectrum of 1-monostearin in the β-crystalline form published by Chapman (22), who showed that the profound effect of crystallinity upon the in-

[3] The chromatographic adsorption affinity of neutral lipides is predominantly determined by the number of free hydroxyl groups (12), and hence the elution sequence for glycerides is tri-, di-, monoglycerides (13). This order manifests itself by the increasing appearance of an OH band between 3500 and 3350 cm.$^{-1}$ in the infrared spectra of subsequent eluates (Fractions Ic to If).

frared spectra of glycerides can be used to differentiate between the various polymorphic forms.[4] Infrared spectra of glycerides taken from solutions show broad and relatively unspecific absorptions not suitable for the identification of individual compounds, while the spectra of crystalline films (Fig. 1, Spectra 1 to 3), especially of the most stable β form, present a striking fine structure which consists of numerous closely spaced sharp bands characteristic for each compound. Chapman's data also show that the spectra of crystalline monoglycerides esterified on carbon 1 differ grossly from those of the corresponding 2 isomers, whereas variations in the chain length of the fatty acid residues within either series of isomers produce only relatively small spectral changes in certain regions. In view of these correlations it can be concluded that Smith's Compound D is a 1-monoglyceride of either stearic or palmitic acid.

Although their glyceride nature was confirmed by hydrolysis, Compounds A and D were not further investigated by chemical methods. Fractions Id and IIb and IIc, which had an adsorption affinity corresponding to that of a diglyceride, had an infrared spectrum similar to the one of Compound C published by Smith *et al.* (18). The chemistry of this compound, which represents 20 to 30 per cent of the acetone-soluble fat, was investigated in detail. It will be referred to as Smith's Compound C.

Isolation of Smith's Compound C and α-Monoglyceride of Mycolic Acid—For further purification the fractions containing Compound C (Id and IIb and IIc) were extracted exhaustively with boiling methanol. Upon being cooled, a white crystalline precipitate separated which was filtered on Whatman paper No. 41-H and dried *in vacuo*. The infrared spectrum of the crystalline precipitate (Fig. 1, Spectrum 1) was identical with that of Smith's Compound C. The methanol-insoluble residue remaining after extraction with boiling methanol was dissolved in ether and precipitated with excess methanol. After being filtered and dried, it yielded a white powder which was identified by its infrared spectrum and melting point with the α-monoglyceride of mycolic acid previously isolated from Wax C and from Dubos' "toxic lipide" (12, 13).

Chemistry of Smith's Compound C—After two crystallizations from hot methanol, Compound C had the following characteristics: m.p. 67–68°; acidity 0; $[\alpha]_D^{27}$ 0° ± 0.2° (c = 5 per cent in $CHCl_3$).

Saponification—100 mg. of Compound C were saponified with KOH in moist isopropyl alcohol, as described for cord factor (3). Upon cooling, a heavy gelatinous precipitate formed. Addition of 10 ml. of H_2O gave a

[4] Dr. H. M. Randall informed us that this has been confirmed in an unpublished study on the effect of crystallinity on the infrared spectra and x-ray diffraction patterns of glycerides. We wish to thank Dr. Randall for sending us copies of his spectra of known glycerides and for his helpful comments.

clear soapy solution. No material could be extracted from the alkaline solution with ether.

Isolation of Fatty Acids—After acidifying the reaction mixture with HCl, the ether extracts yielded a waxy residue which was crystallized twice from hot methanol. This yielded 55 mg. of a white microcrystalline powder with the following properties: m.p. 58–60°; acid equivalent 276; C 75.31, H 12.53.

Infrared Spectrum—The infrared spectrum of the acidic material taken as a melt showed the characteristic absorptions of long chain fatty acids

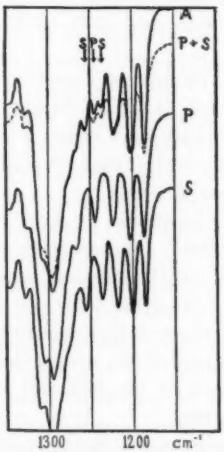

FIG. 2. Infrared spectra of progression bands of fatty acids (taken from melts). Spectrum A, fatty acids from alkaline hydrolysis of Compound C; Spectrum P + S, equimolar mixture of palmitic and stearic acid; Spectrum P, palmitic acid; Spectrum S, stearic acid.

in the solid state. According to Jones *et al.* (23) and Meiklejohn *et al.* (24), saturated straight chain fatty acids can be identified by the number and positions of the progression bands between 1350 and 1150 cm.$^{-1}$. In Fig. 2 the progression bands of the acidic material from alkaline hydrolysis of Compound C (Spectrum A) are compared with those of palmitic acid (Spectrum P), stearic acid (Spectrum S), and an equimolar mixture of the two acids (Spectrum P + S, broken line). An analysis of these spectra shows that Spectrum A contains the progression bands of both palmitic and stearic acid and is identical with Spectrum P + S, indicating that the ether-soluble saponification product represents an equimolar mixture of palmitic and stearic acid.

This conclusion is corroborated by the data from titration and ele-

mentary analysis,[5] both indicating an equimolar mixture of palmitic and stearic acids. Acid equivalent: theory, 270; C 75.50, H 12.67; acid equivalent: found, 276; C 75.31, H 12.53.

Isolation of Water-Soluble Fragment—The aqueous portion of the hydrolysate gave negative Molisch and sulfuric acid tests (25) for carbohydrate. The solution was deionized by being filtered through Amberlite MB-3 and taken to dryness *in vacuo*. The resulting colorless viscous liquid was identified as glycerol by infrared spectroscopy and paper chromatography (butanol-acetic acid-water, 25:6:25). Spraying with aniline phthalate gave no spots and development with $AgNO_3$ and ethanolic NaOH (26) revealed only one spot with an R_F value identical to that of the glycerol control.

Reduction with $LiAlH_4$—75 mg. of Compound C were reduced in anhydrous ether with 93 mg. of $LiAlH_4$. 58 mg. (77 per cent) of an ether-soluble neutral reaction product were isolated in the usual way. It had a melting point of 51–52° and its infrared spectrum had the typical absorptions of long chain paraffinic alcohols.

Acetylation—60 mg. of Compound C were treated with excess acetic anhydride in benzene-pyridine. The reaction product was not soluble in hot methanol in contrast to the parent compound. Purification was effected by reprecipitation from ether with excess methanol. The product (55 mg.) was a white microcrystalline powder with a melting point of 49–50°. The infrared analysis of the acetyl derivative (Fig. 1, Spectrum 2) showed the disappearance of the free OH band of Compound C, indicating complete acetylation.

Chemical Structure—These results indicate that Smith's Compound C is a mixed diglyceride of stearic and palmitic acids corresponding to the formula $C_{37}H_{72}O_5$. Further evidence was provided by elementary analysis of Compound C and its acetyl derivative.

Compound C, $C_{37}H_{72}O_5$.	Calculated.	C 74.44, H 12.16
	Found.	" 74.33, " 12.09
Acetyl derivative, $C_{39}H_{74}O_6$.	Calculated.	" 73.30, " 11.67
	Found.	" 73.05, " 11.55

Of the three isomers corresponding to this composition, only two have melting points similar to Compound C, namely 1-stearo-2-palmitin (68.5–69.5°) and 1-stearo-3-palmitin (71.0–71.5°) (27, 28). The non-reactivity of Compound C with tritylchloride (27) suggests that it is 1-stearo-3-palmitin.

[5] We are indebted to Ciba Pharmaceutical Products, Inc., Summit, New Jersey, for carrying out the microanalyses.

Group II. Phosphoglycolipides

Of all the lipide fractions of *M. tuberculosis*, the purification of the phosphatides has proved to be most difficult. Extensive studies in the past, summarized by Asselineau and Lederer (15), have shown that they are of a complex nature, and quite different from the phosphatides commonly found in animals and plants. Hydrolytic degradation always liberated a considerable proportion of carbohydrates, notably inositol, and some amino acids. It seems more appropriate, therefore, to use the term "phosphoglycolipides" in describing these fractions.

Lederer and coworkers have succeeded by chromatography in partitioning the crude phosphoglycolipides into fractions of different composition (29, 30). The homogeneity and structure of most of these fractions, however, have not been rigidly established.

The presence of phosphoglycolipides in some of the fractions obtained after chromatography of the acetone-soluble fat was detected by infrared spectroscopy and phosphorus assay (31). The phosphorus-containing material was concentrated in Fractions IIa and IIe. Further purification was achieved by precipitation with acetone from ether or benzene and by rechromatography of the acetone-insoluble precipitates. The phosphorus content of the resulting fractions varied between 1 and 3 per cent. The details of these procedures and analytical data on the products will be reported later. However, since this paper is concerned with a characterization by fractionation and infrared spectroscopy of the main components of the acetone-soluble fat, a short discussion of the infrared spectra of the phosphoglycolipides is included here.

Infrared Spectra—The infrared spectra of non-bacterial phospholipides have been studied by a number of investigators (32–34), who showed that the different classes of the lower molecular weight phospholipides give characteristic absorption spectra useful for identification (32). A characteristic feature of phospholipide spectra between 1235 and 1200 and 1100 and 1000 $cm.^{-1}$ is the presence of at least three strong bands, probably associated with covalent phosphate. As evident from a comparison in Table III, the bands of a representative phosphoglycolipide sample agree well in this region with the corresponding phosphate absorptions of chemically defined phospholipides.

However, apart from this similarity with respect to the phosphate absorptions, the phosphoglycolipides from *M. tuberculosis*, because of their complex nature and high carbohydrate content, give usually less clearly defined infrared spectra than the known phospholipides of low molecular weight. Spectrum 1, shown in Fig. 3, is representative for a large number of partially purified phosphoglycolipide fractions prepared

from the acetone-soluble fat. Similar spectra were obtained with Yamamura's cavity-inducing proteolipide (35) (Fig. 3, Spectrum 2) and Nègre's antigène méthylique (36) (Fig. 3, Spectrum 3). As can be seen, the bands of the covalent phosphate group and the C—O stretching absorptions of the carbohydrate part overlap between 1200 and 1000 cm.$^{-1}$, causing strong, broad, and only partially resolved absorptions in this region.

In addition to the strong phosphate and C—O absorptions between 1200 and 1000 cm.$^{-1}$, the spectra of these preparations show an intense ester C=O stretching band between 1750 and 1700 cm.$^{-1}$. The shape and position of this band vary, indicating different degrees of hydrogen bond-

TABLE III

Main Absorption Bands in Infrared Spectra of Phospholipides Due to Covalent Phosphate Group

Compound*	Frequency of absorption maxima in cm.$^{-1}$		
L-α-Glycerophosphorylcholine ($CdCl_2$ salt)	1200	1090	1060†
Lecithin	1235	1090	1060
Sphingomyelin	1230	1090	1060
Cephalin	1220	1080	1010
Acetal phospholipide	1220	1080	1010
Phosphoglycolipide Fraction IIe, acetone-insoluble (Fig. 3, Spectrum 1)	1235	1110	1060

* Data on the first five compounds were taken from Marinetti and Stotz (32).

† The absorption maxima shown in the brackets form a broad doublet.

ing. The ester C—O—C linkages absorb near 1240, thus contributing to the strong covalent phosphate band in this region (32). The spectra of some of these preparations show a pair of rather weak bands near 1650 and 1550 cm.$^{-1}$ characteristic of —CONH—, suggesting the presence of small amounts of peptide components. The high carbohydrate content (10 to 40 per cent) in all of these samples accounts for the strong broad band at 3350 cm.$^{-1}$ characteristic for bonded OH. Despite the general similarity of the spectra of the various phosphoglycolipide fractions, there are a number of minor but distinct differences in band positions and in number and relative intensities of the maxima. However, since these fractions are not homogeneous, no useful correlations for more detailed structural interpretations can be made. Yet, in a qualitative way these infrared spectra are sufficiently characteristic to distinguish the phosphoglycolipides from the lower molecular weight lipides obtained during the purification of the crude extracts.

DISCUSSION

Correlations between infrared spectra and chemical structure established in the course of this and previous studies make it possible to classify or

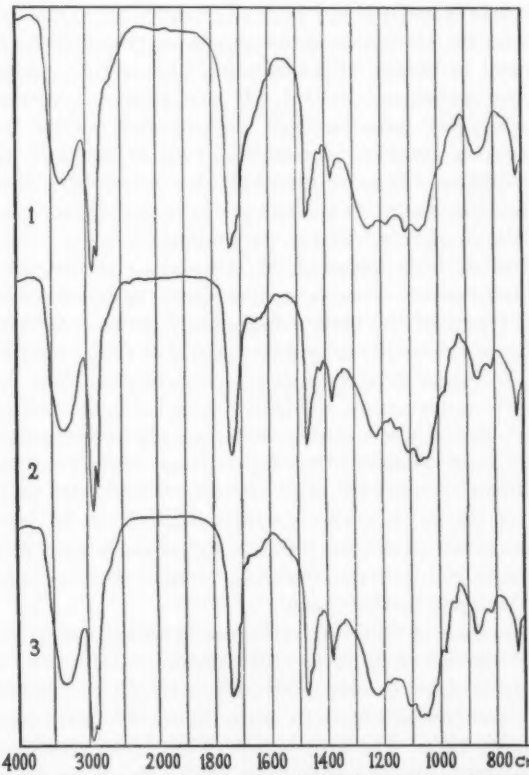

FIG. 3. Infrared spectra of phosphoglycolipides (taken from Nujol mulls). Spectrum 1, phosphoglycolipide fraction from chromatography of acetone-soluble fat; Spectrum 2, Yamamura's cavity-inducing proteolipide; Spectrum 3, Nègre's antigène méthylique.

identify by rapid spectroscopical screening the main lipide components obtained by various procedures of extraction and fractionation. In terms of these findings the lipides of *M. tuberculosis* (Brévannes) can be divided into two main categories: I, complex phosphoglycolipides; II, lower molecular weight lipides.

I. Complex Phosphoglycolipides—Most biologically active lipides belong to the first group which includes the lipopolysaccharides (Wax D) with

sensitizing action (37) and various preparations of phosphoglycolipides such as Nègre's antigène méthylique (36), Yamamura's cavity-inducing proteolipide (35), and Pound's wax fraction (38) with adjuvant properties. The degree of homogeneity and the chemical structure of these preparations remain largely unknown.

It was found that the phosphorus-containing glycolipides are not completely removed by the initial precipitation with acetone during the preparation of the acetone-soluble fat. It is a common experience in the purification of highly complex lipide mixtures that solvent fractionation often fails to give complete separations, even of fractions with widely differing solubilities. This is apparently due to strong molecular interactions. Likewise, many compounds are extracted by solvents in which they have little if any solubility in the purified state.

Biological work with lipides of *M. tuberculosis* in the past has often been based on the nature of the extracting solvent system for distinguishing the chemical nature of the various biologically active materials from one another. The above results emphasize again that this is a poor criterion.

II. Lower Molecular Weight Lipides—In contrast to these preparations of a relatively complex nature, the native lipides of lower molecular weight have now been well characterized spectroscopically and chemically. Their characteristic, clearly defined infrared spectra and distinct chromatographic absorption affinities facilitate their separation from the more complex components of higher molecular weight. They seem to be devoid of toxicity or antigenic properties with the exception of cord factor which, from its chemical structure and molecular weight, is to be regarded as an intermediate between the two classes.

A large proportion of the lower molecular lipides are esters of saturated straight and branched chain fatty acids (ranging in size from C_{16} to C_{88}) with various hydroxylated compounds such as glycerol, long chain alcohols (phthiocerol), and aromatic hydroxy ethers (probably similar to Anderson's leprosols (12, 39, 40)). The distribution of the latter type of compounds appears to be strain-specific, while most of the glycerides (Smith's Compounds A, C, and D) have been isolated from all the strains studied, including H37Rv and BCG as well as a number of atypical and saprophytic variants (41).

In agreement with Smith and his coworkers it was found that mono-, di-, and triglycerides are relatively abundant in the acetone-soluble fat fraction of the methanol-ether extracts. This parallels our previous findings on the composition of Wax C (12, 13). The glycerides of the acetone-soluble fat, however, differ from those in Wax C by the predominame of shorter chains (C_{16} and C_{18}) in their constituent fatty acids.

This accounts for their preferential extraction with methanol-ether. The presence of some α-monoglyceride of mycolic acid in the acetone-soluble fat shows that there is some overlapping between the latter fraction and Wax C. The observed abundance of glycerides, already reported by Smith, is in contrast to results obtained by Anderson and coworkers (42), as well as by Aebi *et al.* (6).[6] According to these authors, trehalose serves in place of glycerol in these lipides. We were unable to detect esters of trehalose in the acetone-soluble fat. So far, trehalose-6,6′-dimycolate (cord factor) is the only chemically pure native glycolipide that has been isolated from *M. tuberculosis*. The isolation of trehalose in hydrolysates of the acetone-soluble fat reported in the literature suggested the occurrence of structural analogues of cord factor in which trehalose was esterified with shorter fatty acids (11). The present findings fail to substantiate our earlier hypothesis. It is possible, therefore, that the different results reported in those earlier investigations were due either to the presence of cord factor in these extracts or, as already mentioned, to differences in the production of the bacterial cultures.[7]

SUMMARY

The chemical composition of the acetone-soluble fat of *Mycobacterium tuberculosis*, strain Brévannes, has been examined by infrared spectroscopy after chromatographic partitioning. On the basis of their spectral and chemical characteristics the separated native lipide constituents of the acetone-soluble fat fraction were found to belong to one of the following three classes of lipides: I, mono-, di-, and triglycerides; II, complex phosphoglycolipides; and III, 1,4-naphthoquinone derivatives.

One of the main components of the first group, Smith's Compound C, was identified as a mixed diglyceride of stearic and palmitic acids. The infrared absorptions and solubility properties of the complex phosphoglycolipides were investigated, methods for their separation from the phosphorus-free lipides of lower molecular weight outlined, and their relations to various

[6] The influence of the glycerol-containing culture medium has been suggested as a possible explanation for the observed high glyceride content (12).

[7] The extracts described in this paper were prepared from cultures used for the production of cord factor. In contrast to the authors quoted who extracted with ethanol-ether (1:1), we used methanol-ether (2:1), in order to avoid in this first step the extraction of cord factor which is subsequently extracted with chloroform. Since we found that cord factor has a certain solubility in ethanol-ether (1:1), it is likely that at least part of the trehalose found by those previous investigators was derived from cord factor. On the other hand, strain differences could hardly account for the differences in glyceride content, since one of the strains used by Smith *et al.* (H37Rv) was identical with the strain employed by Anderson, and ours (Brévannes) with the one used by Aebi.

biologically active preparations discussed. The naphthoquinone derivatives are the subject of a subsequent paper.

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THE CHEMISTRY OF THE NATIVE CONSTITUENTS OF THE ACETONE-SOLUBLE FAT OF MYCOBACTERIUM TUBERCULOSIS (BREVANNES)*

II. ISOLATION AND PROPERTIES OF A NEW CRYSTALLINE NAPHTHOQUINONE DERIVATIVE RELATED TO VITAMIN K_2

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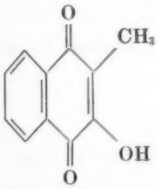
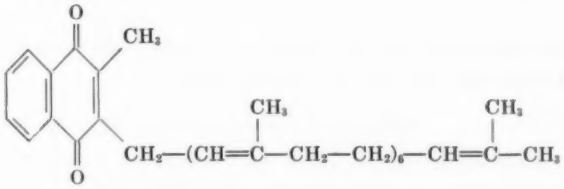
(Received for publication, February 3, 1958)

The chromatographic fractionation of the acetone-soluble fat of *Mycobacterium tuberculosis* (Brévannes) and the characterization of its main native constituents have been reported in Paper I (1). It was shown that the acetone-soluble fat yielded some fractions with typical naphthoquinone absorptions in addition to the glycerides and complex phosphoglycolipides which have been described in detail in the previous study. The occurrence of 1,4-naphthoquinone derivatives in the acetone-soluble fat has already been demonstrated by Anderson and Newman with the isolation of phthiocol (I) (2). More recently, Snow and coworkers (3) isolated from similar extracts of an unspecified strain of *M. tuberculosis* a polyisoprenoid 1,4-naphthoquinone derivative, apparently closely related to vitamin K_2 (II) (4).



(I)



(II)

The present paper describes the isolation of two naphthoquinone derivatives obtained during the chromatography of the acetone-soluble fat. One of these compounds, probably identical with Snow's oil fraction, has

*This investigation was supported by research grants No. E-1500 and No. E-1297 from the Division of Research Grants of the National Institutes of Health, Public Health Service.

A portion of this work was carried out at the Public Health Research Institute of the City of New York, Inc., New York, New York.

been obtained in the crystalline state. Some physicochemical data of this compound and structural deductions derived from detailed spectroscopic studies, especially in the infrared region, are reported below.

EXPERIMENTAL

Details on the preparation and chromatography of the acetone-soluble fat have been given in Paper I (1). Infrared absorption spectra characteristic for naphthoquinones were associated with two fractions of the acetone-soluble fat, an oil eluted from silicic acid with petroleum ether-benzene (Fraction Ib) and a solid eluted with ether-methanol (Fraction Ih).[1] In order to facilitate the description, and because of their

TABLE I

Chromatogram I. Chromatography of 1.10 Gm. of Fraction I Dissolved in Petroleum Ether, Adsorbed on 50 Gm. of Magnesium Silicate-Celite

Fraction No.	Eluted with	Amount	Aspects of material
		mg.	
1	250 ml. petroleum ether	720.5	Light yellow oil
2	50 " " "	Trace	
3	300 " " " ether-benzene, 1:1	143.5	Light brown soft solid
4	300 ml. ether-benzene, 1:1	1.9	
5	100 " ether-methanol, 9:1	Trace	
Total.....		865.9	
Recovered, %....		73	

relationship to the K vitamins, these two fractions will be referred to as Compounds K_x and K_y, respectively.

Isolation of Compound K_x in Crystalline Form

Fraction Ib, which was a light orange oil, was further purified by chromatography on magnesium silicate-Celite (Table I); two distinct fractions were obtained. The first fraction was a lemon yellow oil (66 per cent of the starting material) with the spectral characteristics of a naphthoquinone (Fig. 1, Spectrum 3); the second, a light brown soft solid consisted mainly of triglycerides contaminated with some naphthoquinones. The yellow oil (K_x) became orange on standing and, after several weeks at room temperature, a crop of bright yellow crystals separated from the oil. The crystals formed small balls which clumped together to large aggregates of the consistency of a hard wax. These characteristics and the solubility

[1] The chromatographic fractions referred to are listed in Table I of Paper I of this series.

of the material resembled closely the properties reported for vitamin K_2 by Doisy and coworkers (5).

Physicochemical Properties—The physicochemical data of a sample of 20 mg. of purified Compound K_x obtained after two crystallizations, the

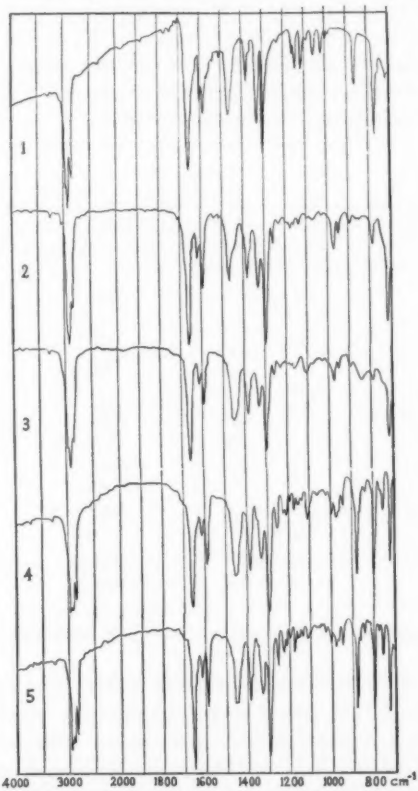


FIG. 1. Infrared spectra of 1,4-naphthoquinone derivatives. Spectrum 1, 1,4-naphthoquinone (Nujol mull); Spectrum 2, vitamin K_1 (smear); Spectrum 3, Compound K_x (oil); Spectrum 4, crystalline Compound K_x (resolidified melt); Spectrum 5, vitamin K_2 (resolidified melt).

first from acetone-methanol and the second from acetone, are listed in Table II, together with those of Snow's compound (3) and vitamin K_2 (5).[2] The ultraviolet absorption spectrum of Compound K_x in isooctane

[2] After this paper went to press, Dr. Isler informed us of his revised formula for vitamin K_2. The new formula is based on the synthesis of vitamin K_2 and differs from the original formula by having 1 additional isoprenoid unit in the side chain (22). The revised formula and C,H values are given in this paper.

was identical in shape and relative intensities of the peaks with the spectra of the vitamins K_1 and K_2 (6). The absorbance values, however, were lower than either, being virtually identical with those reported by Snow and coworkers (3) for a highly purified oil fraction isolated by chromatography from the native lipides of *M. tuberculosis*. As seen from Table II, the analytical data[3] of crystalline Compound K_x are in closest agreement with those of Snow's compound. The melting point of Compound K_x (58–59°) was somewhat higher than that of vitamin K_2 (53.5–54.5)[4] and a mixture of the two substances melted at 49–53°.

TABLE II

Analytical Data of Compound K_x, Vitamin K_2, and Snow's Compound

	Compound K_x	Snow's Compound (3)	Vitamin K_2 (5, 6)
	$E_{1\ cm.}^{1\%}$	$E_{1\ cm.}^{1\%}$	$E_{1\ cm.}^{1\%}$
Absorption maxima			
243 mμ	226	227	287
248 "		241	
249 "	240		305
260 "	218	220	275
269 "		216	
270 "	209		275
	°C.	n_D^{25}	°C.
M.p.	58–59	1.5286 (oil)	53.5–54.5
	per cent	*per cent*	*per cent*
C	85.24	85.10	85.13
H	10.26	10.27	9.94

The figures in parentheses represent bibliographic references.

Infrared Studies—The infrared spectrum of the crystalline Compound K_x (Fig. 1, Spectrum 4) was found to be qualitatively identical with that of vitamin K_2 (Fig. 1, Spectrum 5). In order to obtain more detailed information about the structure of Compound K_x and the significance of its infrared absorptions in relation to the other analytical findings, the spectral characteristics of similar compounds of known constitution were investigated.

In a first approximation, the infrared spectrum of vitamin K_2 can be analyzed by regarding it as a composite spectrum made up of the absorptions contributed by the 1,4-naphthoquinone nucleus and the polyiso-

[3] We are indebted to Ciba Pharmaceutical Products, Inc., Summit, New Jersey, for carrying out the microanalyses.

[4] The author wishes to express his thanks to Dr. E. A. Doisy for a most generous gift of this rare vitamin.

prenoid side chain. This approach, though neglecting the secondary vibrational effects which result from the mutual interactions of these two component parts, proved to yield useful correlations for the interpretation of the main spectral characteristics of vitamin K_2.

Absorption of Naphthoquinones

The spectra of naphthoquinones are relatively unexplored and only few correlations have been established with certainty (Bellamy (7), p. 129). Most studies have been concerned with the influence of substitutions in the ring on the C=O stretching absorption near 1660 cm.$^{-1}$ (8, 9).

TABLE III

Characteristic Infrared Absorption Spectra of 1,4-Naphthoquinone Derivatives

Compound	Frequency of absorption maxima in cm.$^{-1}$				
	C=O	C=C (aromatic)		C—O (C=C)	
	Band a	Band b	Band c	Band d	Band e
1,4-Naphthoquinone	1660	1600	1585	1330	1300
Vitamin K_1	1660	1618	1595	1330	1295
" K_2	1655	1615	1590	1330	1295
Compound K_x	1655	1613	1590	1330	1295
" K_y	1650	1613	1590	1330	1295
Phthiocol	1655, 1640*	1625*	1583	5 bands	1350–1210
Lapachol	1655, 1640	1625*	1587	5 "	1350–1210

* Band visible as shoulder.

A comparison of the spectrum of 1,4-naphthoquinone with the spectra of a series of derivatives[6] (Figs. 1 and 2) shows that all of them contain strong bands in the 1660 to 1590 and 1350 to 1200 cm.$^{-1}$ regions (Table III). By means of these absorptions the compounds listed in Table III can be divided into two groups: Group I, 1,4-naphthoquinone and its alkyl derivatives (Fig. 1); Group II, derivatives of 1,4-naphthoquinone having a hydroxyl group attached to the quinone ring (Fig. 2). The compounds of the first group exhibit in these regions a characteristic and constant pattern of five sharp bands (Table III, Bands a to e) which are similar in relative intensity and position.

The first set of three bands (Bands a to c) consists of a very strong band near 1660 (Band a) due to the carbonyl stretching and common to all

[6] I am indebted to Dr. L. F. Fieser for samples of phthiocol and lapachol and to Hoffmann-La Roche, Inc., for a gift of vitamin K_1.

quinones, a weaker band near 1615 (Band b), followed by a stronger band near 1590 (Band c). The latter two bands arise most probably from the aromatic C=C stretching. This would be in agreement with earlier observations that the 1600 cm.$^{-1}$ band, normally occurring in aromatic

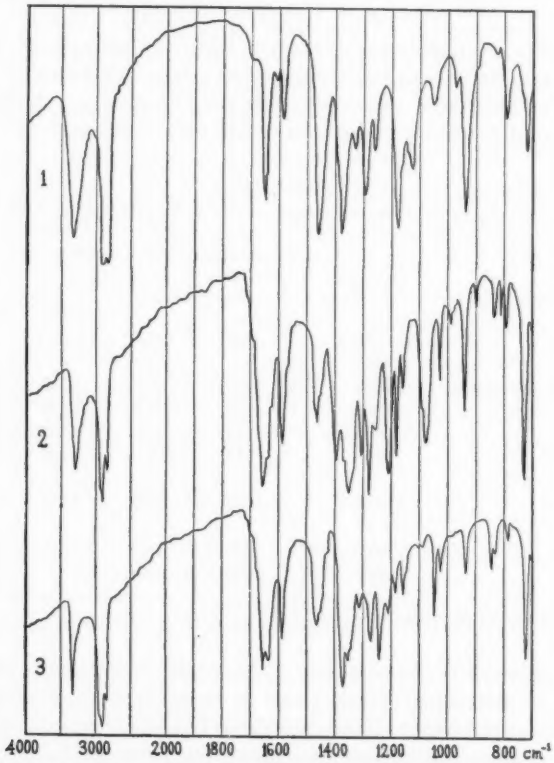


FIG. 2. Infrared spectra of 1,4-naphthoquinone derivatives (taken from Nujol mulls). Spectrum 1, Compound K_y; Spectrum 2, phthiocol; Spectrum 3, lapachol.

compounds, is split into two bands in naphthalenes and other polycyclic aromatics (Bellamy (7), p. 61) and that the intensity of the lower band is considerably enhanced when a carbonyl or other unsaturated group is attached directly to the ring.

The second set of absorptions common to all compounds of the first group consists of two bands (Bands d and e), one of medium intensity at 1330 (Band d) followed by a second very strong band near 1300 cm.$^{-1}$ (Band e). The nature of these naphthoquinone bands in the C—O region

around 1300 cm.$^{-1}$ has not been elucidated, but it is likely that they arise from an interaction between the carbonyl group and the aromatic C=C bonds.

The second group of naphthoquinone derivatives shows a less regular absorption pattern. Apparently, the presence of a hydroxyl group in the ring results in a pronounced alteration of the absorptions found to be characteristic for 1,4-naphthoquinone and its alkyl derivatives. Thus, instead of the relatively weak Band c near 1615 cm.$^{-1}$ in the spectra of the first group, the spectra of the hydroxyl derivatives show two rather strong absorptions at 1625 and 1640 cm.$^{-1}$, either fully separated or visible as shoulders. It is possible that the additional band at 1640 cm.$^{-1}$ results from a splitting of the C=O band due to hydrogen bonding, while the 1625 cm.$^{-1}$ band represents a shift of the 1615 cm.$^{-1}$ C=C absorption towards higher frequencies. In the 1300 cm.$^{-1}$ region the derivatives with a hydroxyl group attached to the ring also present a more complex system of absorptions than the first group. These bands are probably due to C—O stretching modes of the carbonyl and hydroxyl groups and their possible interactions with the aromatic C=C vibrations.

It may be concluded from this study that alkyl-substituted 1,4-naphthoquinones can be identified by a set of five well defined, generally strong bands which are independent of the nature of the alkyl substituents and hence characteristic for the 1,4-naphthoquinone nucleus.

Polyisoprenoid Absorptions

Isler *et al.* (10) published the spectra of natural and synthetic squalene, and Saunders and Smith (11) made a detailed examination of the infrared absorptions of rubber polyisoprenes. By comparing amorphous material with different crystalline modifications and by using the technique of oriented crystalline films together with polarized radiation, Saunders and Smith were able to deduce correlations between infrared absorption spectra and crystalline structure. They found that crystallinity has a pronounced effect upon the absorptions between 900 and 750 cm.$^{-1}$. These correlations are listed in Table IV, together with the absorptions observed in the spectra of squalene, vitamin K_2, and Compound K_x as oil and in the crystalline state. It follows from these data that squalene and Compound K_x in its non-crystalline oil form have an amorphous cis polyisoprenoid chain, while vitamin K_2 and the crystalline Compound K_x show the bands characteristic for the planar transconfiguration of crystalline polyisoprene.

Structure of Compounds K_z and K_y

It has been established by infrared studies that Compound K_x is a 1,4-naphthoquinone with a polyisoprenoid side chain, similar to vitamin

K_2. The somewhat higher carbon content and melting point of our crystalline preparation as well as the depression of the melting point when mixed with vitamin K_2 lend support to the proposal of Snow *et al.* From their studies with a similar oil fraction they suggested that the native naphthoquinone derivative from *M. tuberculosis* is a homologue of vitamin K_2 containing two additional isoprenoid units in the side chain (4). Quantitative considerations of the absorbance values in the ultraviolet as well as

TABLE IV

Characteristic Infrared Absorption Spectra of Crystalline and Amorphous Polyisoprenes

Compound	Frequency of absorption maxima* in cm.$^{-1}$			Crystalline structure
	CH_3 (wag)	C—H (wag)	CH_2 (rock)	
α-Gutta-percha (11).....	883 (m.), 864 (s.)	803 (s.)		Twisted trans crystalline
β-Gutta-percha (11).....	876 (s.)	796 (s.)	752 (w.)	Planar trans crystalline
Vitamin K_2.....	876 (s.)	796 (s.)	753 (w.)	
Crystalline Compound K_x.....	878 (s.)	797 (s.)	753 (w.)	
Amorphous gutta-percha (11).....	890 (w.)	836 (s.)		Cis
Hevea (11).....	885 (w.)	845 (s.)		Amorphous
Squalene (10).....	885 (m.)	835 (s.)		
Amorphous Compound K_x (oil).....	890 (w.)	840 (m.)		

* Estimated relative intensity of absorption bands is expressed by the symbols, s. = strong, m. = medium, w. = weak. "Wag" and "rock" refer to the mode of vibration which causes absorption.

in the infrared regions are also consistent with the assumption of a longer side chain in Compound K_x. Thus, on the basis of the ultraviolet absorption spectra, the side chain can be calculated to contain 7 to 8 isoprenoid units. Similarly, a closer comparison of the infrared spectra of vitamin K_2 and Compound K_x reveals distinct and reproducible quantitative differences in the relative absorption intensities of a number of bands. Notably, the CH_2 and C—CH_3 absorptions near 1450 and 1380 cm.$^{-1}$, respectively, are somewhat stronger in the spectrum of Compound K_x than the corresponding bands of vitamin K_2 when measured against the naphthoquinone bands at 1590 and 1330 cm.$^{-1}$ as internal standards, indicating again the presence of a longer side chain in Compound K_x. Preliminary

x-ray diffraction studies[6] of the two compounds showed similar powder patterns with essentially identical spacings, but marked differences in the relative intensities of the bands. An unequivocal differentiation by this technique, however, would require a complete analysis of a single crystal which was not available.

When examined under the microscope, the crystalline Compound K_x appeared less transparent and morphologically not as well defined as samples of crystalline vitamin K_2. Moreover, the color of the vitamin K_2 crystals has a greenish tinge, whereas the Compound K_x powder was whitish yellow. Hence, the possibility that the reported observations and analytical data are somewhat influenced by small amounts of impurities in Compound K_x, causing incomplete crystallization, cannot be ruled out.

Compound K_y, obtained as a yellow amorphous powder after triturating Fraction Ih with benzene, was not further purified. In contrast to Compound K_x, Compound K_y was very soluble in methanol and slightly soluble in ether. These properties indicate that Compound K_y is more polar than Compound K_x and lacks the long hydrocarbon side chain present in Compound K_x. The infrared spectrum of Compound K_y shows a strong OH band but none of the characteristic shifts in band positions observed for ring-substituted hydroxy derivatives of 1,4-naphthoquinone. These findings suggest that Compound K_y is either a precursor or a degradation product of Compound K_x, yet different from phthiocol in that its hydroxyl group is attached to a short side chain rather than to the quinone ring.

DISCUSSION

The presence of a vitamin K factor in extracts of *M. tuberculosis* has been postulated by Fieser *et al.* (12) in order to explain the origin of phthiocol isolated by Anderson in alkaline hydrolysates of the acetone-soluble fat. This was later confirmed by Snow *et al.*, who isolated a highly unsaturated oil resembling vitamin K_2 and yielding phthiocol after alkaline hydrolysis. It appears that our crystalline Compound K_x is identical with Snow's oil fraction and represents the postulated native phthiocol precursor. Infrared spectroscopy furnished definitive proof that Compound K_x contains a polyisoprenoid side chain.

In the light of this result it seems possible now to understand the hitherto obscure origin and chemical nature of a group of unsaturated aliphatic compounds isolated by Aebi *et al.* from the unsaponifiable portion of certain fractions of the acetone-soluble fat (13). The C—CH_3 and C=C

[6] The author wishes to thank Mr. W. Kehl, Gulf Research and Development Company, Harmarville, Pennsylvania, for the courtesy of preparing the x-ray powder diagrams.

values reported for these compounds of the approximate composition $C_{26}H_{44}O_2$, $C_{26}H_{42}O_3$, $C_{26}H_{44}O_5$ are indicative of a polyisoprenoid structure, suggesting that they are partially oxidized degradation products which originate from the side chain of the native naphthoquinone derivative. The isolation of esters of phthalic acid by Aebi *et al.* in the same extracts could be similarly explained.

Extracts with vitamin K activity have been prepared previously from a number of microorganisms. Despite the absence of sufficient data for precise identification, it is generally stated in the literature that microorganisms produce the polyisoprenoid vitamin K_2 in contrast to the phytyl derivative vitamin K_1 found in the chloroplasts of green plants (14). Although the precise role of the K vitamins in the cellular metabolism is still obscure, evidence is accumulating that they are important cofactors in electron transfer reactions and probably involved in photosynthetic (15–17) and oxidative (18, 19) phosphorylation. The recent isolation of a new quinone from electron transfer particles of beef heart mitochondria (20) should also be noted in this connection.

It is tempting to speculate that the structural variations of the K vitamins, of which the present Compound K_x is a new example, reflect differences in the nature of the corresponding electron transfer systems. The observation of Fieser and Fieser (21) that the value of the oxidation-reduction potential of naphthoquinone derivatives is markedly influenced by the nature of the substituents appears to support such a view.

SUMMARY

The native parent compound of Anderson's phthiocol was isolated in crystalline form from the acetone-soluble fat fraction of *Mycobacterium tuberculosis* (Brévannes). Physicochemical data and spectroscopic studies in the ultraviolet and infrared regions led to the conclusion that the new compound is a polyisoprenoid derivative of 1,4-naphthoquinone homologous to vitamin K_2, but having a longer side chain.

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METABOLISM OF PROPIONIC ACID IN ANIMAL TISSUES

IV. FURTHER STUDIES ON THE ENZYMATIC ISOMERIZATION OF METHYLMALONYL COENZYME A*

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(Received for publication, January 14, 1958)

It has been shown (1, 2) that the main oxidation pathway of propionic acid in animal tissues proceeds by way of the following steps:

$$\text{Propionate} \xrightarrow[(a)]{\text{ATP,}^1\ \text{CoA}} \text{propionyl CoA} \xrightarrow[(b)]{\text{ATP, } CO_2} \text{methylmalonyl (isosuccinyl) CoA} \xrightarrow[(c)]{} \text{succinyl CoA} \xrightarrow[(d)]{} \text{succinate} \xrightarrow[(e)]{(O_2)} CO_2 + H_2O$$

Step (*c*), *i.e.* the isomerization of methylmalonyl CoA to succinyl CoA, is particularly interesting since it involves the migration of a carboxyl group, a reaction hitherto without precedent in chemistry or biochemistry. The enzyme catalyzing this reaction, methylmalonyl CoA isomerase, is widely distributed in animal tissues and has been partially purified from sheep kidney cortex (2). This paper reports on further work dealing with (1) additional evidence that the immediate product of the enzymatic isomerization of methylmalonyl CoA is succinyl CoA, (2) the stereospecificity of Steps *b* and *c* above, and (3) the mechanism of the isomerization reaction.

Product of Methylmalonyl CoA Isomerization—Previous evidence for the formation of succinyl CoA from methylmalonyl CoA was based on the conversion of succinyl CoA to succinamic acid, by treatment with concentrated NH_4OH, and chromatographic identification of the succinamic acid (2). Further evidence has been obtained by coupling the isomerization of methylmalonyl CoA to a multienzyme system (Reactions 1 through 4)[2] whereby acetoacetyl CoA formed by the specific transfer of

* Aided by grants from the National Institute of Arthritis and Metabolic Diseases (Grant A-529), National Institutes of Health, United States Public Health Service, the American Cancer Society (recommended by the Committee on Growth, National Research Council), the Rockefeller Foundation, and by a contract (N6onr279, T. O. 6) between the Office of Naval Research and New York University College of Medicine.

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[1] The following abbreviations are used: ATP, adenosine triphosphate; CoA, coenzyme A.

[2] The compounds in bold-faced type are the substrates added to the mixture of the four enzymes indicated.

CoA from succinyl CoA to acetoacetate, catalyzed by acetoacetyl-succinic thiophorase (CoA transferase), is cleaved to acetyl CoA in the presence

TABLE I

Methylmalonyl CoA Isomerase-Dependent Synthesis of Citrate by Multienzyme System

The complete system contained, in a volume of 1.5 ml., potassium phosphate buffer, pH 7.4 (50 μmoles), reduced glutathione (10 μmoles), methylmalonyl CoA (1 μmole), potassium acetoacetate (20 μmoles), potassium oxalacetate (20 μmoles), and the following enzymes: methylmalonyl CoA isomerase (2) (Experiments 1 and 2, specific activity 0.36, 2.0 mg. of protein; Experiment 3, specific activity 0.65 c.p.m., 1.5 mg. of protein), CoA transferase (3) (specific activity 1200, 0.3 mg. of protein), thiolase (4) (specific activity 6000, 0.5 mg. of protein), and crystalline condensing enzyme (5) (0.3 mg. of protein). After incubation for 75 minutes at 30°, the samples were deproteinized with trichloroacetic acid and analyzed for citrate by the method of Natelson *et al.* (6).

Experiment No.	Components	Citrate formed
		μmole
1	Complete system	0.32
	No methylmalonyl CoA	0.03
	" isomerase	0.04
2	Complete system	0.36
	No methylmalonyl CoA	0.04
	" isomerase	0.06
3	Complete system	0.41
	No methylmalonyl CoA	0.03
	" isomerase	0.04
	" CoA transferase	0.07
	" thiolase	0.03
	" condensing enzyme	0.03

of CoA and thiolase, and acetyl CoA is converted to citrate in the presence of oxalacetate and condensing enzyme.

(1) **Methylmalonyl CoA** → succinyl CoA (isomerase)

(2) Succinyl CoA + **acetoacetate** → succinate + acetoacetyl CoA (CoA transferase)

(3) Acetoacetyl CoA + **CoA** → 2 acetyl CoA (thiolase)

(4) 2 acetyl CoA + **oxalacetate** + H_2O → 2 citrate + 2 CoA (condensing enzyme)

As shown in Table I, no citrate was formed by the above system in the absence of methylmalonyl CoA or methylmalonyl CoA isomerase. Since CoA transferase is quite specific for succinyl CoA (3), these experiments provide decisive proof that succinyl CoA is the product of enzymatic isomerization of methylmalonyl CoA.

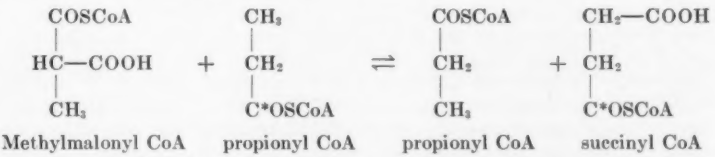
Stereospecificity—Since methylmalonyl CoA has an asymmetric carbon

atom, the preparations obtained by chemical synthesis could consist of a racemic mixture of two stereoisomers of which only one might be attacked by the isomerase. That this is indeed the case is indicated by the fact that, when the enzymatic isomerization of chemically synthesized methylmalonyl CoA is allowed to proceed to completion, under conditions in which the succinyl CoA formed is hydrolyzed to succinate (Steps (*c*) and (*d*), p. 931),[3] only one-half is converted to succinate. On the other hand, methylmalonyl CoA, prepared by enzymatic carboxylation of propionyl CoA (Step (*b*), p. 931), appears to be completely converted to succinate under the same conditions.

Fig. 1 shows the results of two experiments, with different amounts of chemically synthesized methylmalonyl CoA, in which the reaction stopped when about one-half had been converted to succinate. In the experiments of Fig. 2, the extent of enzymatic conversion of methylmalonyl CoA to succinyl CoA was ascertained by paper chromatography and autoradiography of methylmalonic and succinic acids, following alkaline hydrolysis of their CoA derivatives, both before (Samples 2 and 4) and after (Samples 3 and 5) incubation with isomerase of chemically synthesized (Samples 2 and 3) or enzymatically prepared (Samples 4 and 5) C^{14}-labeled methylmalonyl CoA. The composition of the reaction mixtures and other experimental details are given in the legend to Fig. 2. It is apparent that all of the enzymatically prepared, but only about half of the chemically synthesized, methylmalonyl CoA was isomerized.

Mechanism—Evidence for the reversibility of the reaction catalyzed by methylmalonyl CoA isomerase has been presented previously (2). We have now obtained indications that this reaction is a transcarboxylation. In such a reaction the carboxyl group of methylmalonyl (or succinyl) CoA would be transferred to an acceptor molecule of propionyl CoA, which would thereby be converted to succinyl (or methylmalonyl) CoA, with release of 1 molecule of propionyl CoA (Reaction 5). The net reaction would be methylmalonyl CoA $\rightleftharpoons$ succinyl CoA.

(5)



Methylmalonyl CoA — propionyl CoA — propionyl CoA — succinyl CoA

The above mechanism is suggested by experiments showing that C^{14}-labeled succinyl CoA (or methylmalonyl CoA) is formed on incubation of non-labeled methylmalonyl CoA (or succinyl CoA) with C^{14}-labeled pro-

[3] This occurs because the isomerase preparations are contaminated with succinyl CoA deacylase which catalyzes the hydrolysis of succinyl CoA to succinate and CoA.

pionyl CoA (radioactivity indicated by asterisks in Reaction 5) in the presence of isomerase. No label appears in methylmalonyl or succinyl

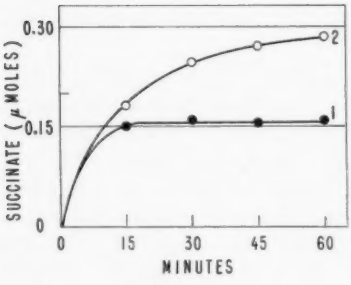


FIG. 1

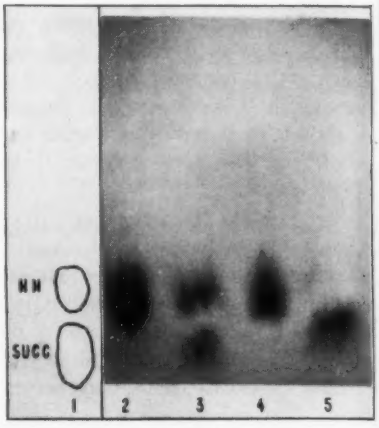


FIG. 2

FIG. 1. Extent of enzymatic conversion of chemically synthesized methylmalonyl CoA to succinate. Succinate was determined (as in isomerase assay (*b*) (2)) at the indicated times in samples containing, in a final volume of 0.9 ml., potassium phosphate buffer, pH 7.4 (50 μmoles), isomerase (specific activity 0.7, 2.2 mg. of protein), and either 0.3 (Curve 1) or 0.6 (Curve 2) μmole of methylmalonyl CoA.

FIG. 2. Autoradiograms showing stereospecificity of enzymatic isomerization of methylmalonyl CoA. All samples contained, in a final volume of 0.9 ml., potassium phosphate buffer, pH 7.4 (50 μmoles), and isomerase (specific activity 0.65, 2.0 mg. of protein). In addition, Samples 2 and 3 contained 1.0 μmole of chemically synthesized methylmalonyl CoA-3-C^{14}, labeled with C^{14} in the methyl group (23,000 c.p.m.), while Samples 4 and 5 contained 1.0 μmole of enzymatically synthesized (from propionyl CoA and $C^{14}O_2$) methylmalonyl CoA-4-C^{14}, labeled with C^{14} in the carboxyl group (23,000 c.p.m.). The CoA thio esters were hydrolyzed with alkali (Samples 2 and 4 at 0 time; Samples 3 and 5 after incubation for 30 minutes at 30°), and the free acids were isolated as described below. Strip 1, markers of succinic (*SUCC*) and methylmalonic (*MM*) acids. After alkaline hydrolysis (15 minutes at room temperature with 0.1 ml. of 1.0 N KOH), each sample was acidified with 0.125 ml. of 4.0 N HCl and extracted three times with 6 ml. portions of ether. The combined ether extracts were added to a tube containing 0.005 ml. of concentrated NH_4OH and evaporated on a steam bath to about 0.1 ml. The methylmalonic and succinic acids were separated by descending paper chromatography (7) in ethanol-water-concentrated NH_4OH (80:15:5) followed by autoradiography.

CoA when the incubation is conducted with non-labeled propionyl CoA in the presence of $C^{14}O_2$. This eliminates the possibility that the isomerization might be the result of *decarboxylation* of methylmalonyl CoA (or succinyl CoA), followed by *recarboxylation* of the resulting propionyl CoA.

Table II illustrates the results of one set of experiments. In Experiment 1a, with isomerase, methylmalonyl CoA (0.6 μmole), and propionyl CoA-1-C^{14} (0.3 μmole, specific radioactivity 69,000 c.p.m. per μmole) a small but definite amount of radioactivity appeared in the succinic acid. With succinyl CoA (0.6 μmole) instead of methylmalonyl CoA (Experiment 2a), radioactivity was found both in succinic and methylmalonic acids. Control experiments showed that no radioactivity appeared in

TABLE II

Isomerization of Methylmalonyl CoA ⇌ Succinyl CoA in Presence of Propionyl CoA-1-C^{14}

The reaction mixtures contained potassium phosphate buffer, pH 7.4 (50 μmoles), and isomerase (specific activity 0.56; free of propionyl CoA carboxylation activity) with 1.8 mg. of protein, in a final volume of 0.9 ml. Other additions as indicated in the text. After incubation for 30 minutes at 30°, the acyl CoA derivatives were hydrolyzed with alkali, and the free acids were isolated by paper chromatography as described in the legend to Fig. 2. The spots of propionic, succinic, and methylmalonic acids were eluted with water, and their radioactivity was determined.

Experiment No.	Additions	Radioactivity of isolated acids		
		Propionic	Succinic	Methylmalonic
		c.p.m.	*c.p.m.*	*c.p.m.*
1a	Methylmalonyl CoA + propionyl CoA-1-C^{14}	4820	900	0
1b	" " + " CoA-1-C^{14}*	5700	0	0
1c	" " + $KHC^{14}O_3$ + propionyl CoA	68	0	0
1d	Propionyl CoA-1-C^{14}	5500	0	0
2a	Succinyl CoA + propionyl CoA-1-C^{14}	5260	760	168
2b	" " + " CoA-1-C^{14}*	5480	0	0
2c	" " + $KHC^{14}O_3$ + propionyl CoA	80	0	0
2d	Propionyl CoA-1-C^{14}	5680	0	0

* The CoA derivative was used but was previously hydrolyzed in 0.1 N KOH (15 minutes at room temperature) and neutralized.

these compounds when either (*a*) propionyl CoA-1-C^{14} was hydrolyzed with alkali (to yield propionate-1-C^{14} plus CoA) prior to incubation (Experiments 1b and 2b) or (*b*) non-labeled propionyl CoA was used and $KHC^{14}O_3$ (2.5 μmoles, specific radioactivity 52,700 c.p.m. per μmole) was present in the incubation mixture (Experiments 1c and 2c).[4] Moreover, there was no radioactivity in the succinic or methylmalonic acid areas of the chromatograms when the incubation mixtures contained propionyl CoA-1-C^{14} but no methylmalonyl CoA or succinyl CoA (Experiments 1d and 2d).

[4] The reason for the appearance of a small amount of radioactivity in the propionic acid in these experiments is unknown.

The low recovery of radioactivity (about 6000 out of 20,700 c.p.m.) may be due in part to volatility of ammonium propionate and in part to incomplete extraction of free acids with ether and incomplete elution from paper. The latter two factors are probably responsible for the small amount of radioactivity recovered in succinic and methylmalonic acids.

To determine the final specific radioactivity of propionyl CoA and succinyl CoA, duplicate incubations were carried out with isomerase, nonlabeled methylmalonyl CoA, and propionyl CoA-1-C^{14}, under conditions similar to those of Experiment 1a of Table II. One of the samples was utilized for the isolation and determination of the radioactivity of the succinic acid as in Table II. In addition, the amount of succinic acid

TABLE III

Specific Radioactivity of Propionyl CoA and Succinyl CoA on Isomerization of Methylmalonyl CoA in Presence of Propionyl CoA-1-C^{14}

Conditions as in Table II (Experiment 1a) but with 0.2 μmole of propionyl CoA-1-C^{14} and methylmalonyl CoA as indicated. Incubation, 15 minutes at 30°.

Experiment No.	Methylmalonyl CoA added	Present at end		Specific radioactivity		
		Propionyl CoA	Succinyl CoA	Propionyl CoA		Succinyl CoA
				Initial	Final	
	μmoles	*μmole*	*μmole*	*c.p.m. per μmole*	*c.p.m. per μmole*	*c.p.m. per μmole*
1a	0.5	0.18	0.06	69,000	49,200	40,600
1b	1.5	0.19	0.08	69,000	46,000	38,100
2a	0.5	0.19	0.07	680	507	496
2b	1.5	0.17	0.09	680	426	413

present was determined with succinoxidase and cytochrome *c* as previously described (2). To the other sample, hydroxylamine (1 mmole) was added to convert the acyl CoA derivatives to the corresponding hydroxamic acids. These were separated by ascending paper chromatography in isoamyl alcohol saturated with 4.0 M formic acid (1); propionhydroxamic acid was eluted with 50 per cent ethanol and assayed for radioactivity, and its amount was determined colorimetrically (8). As shown in Table III, the specific radioactivity of the propionyl CoA and succinyl CoA at the end of incubation (particularly in Experiment 2) was essentially the same. This is what would be expected from Reaction 5 proceeding in the presence of a pool of radioactive propionyl CoA if this compound is bound by the enzyme in a dissociable fashion. The concentration of propionyl CoA did not change, but its specific radioactivity decreased through dilution by non-radioactive propionyl CoA arising from methylmalonyl CoA.

DISCUSSION

Since methylmalonyl CoA contains an asymmetric carbon atom, it would be expected to exist in two stereoisomeric forms. Our results lend support to this view and indicate further (*a*) that the enzymatic carboxylation of propionyl CoA yields only one of the two enantiomorphs of methylmalonyl CoA and (*b*) that it yields the one form which is acted upon by the isomerase. These results are as expected in the light of the stereospecificity exhibited by most enzymes toward their substrates.

As regards reaction mechanism, our results strongly suggest a transcarboxylation mechanism for the enzyme-catalyzed, reversible conversion of methylmalonyl CoA to succinyl CoA as shown in Reaction 5. An analogy is to be found in the reversible conversion of glucose 1-phosphate to glucose 6-phosphate catalyzed by phosphoglucomutase, a reaction which appears to involve the migration of a phosphate group from C_1 to C_6 of glucose or vice versa. This reaction is known (9, 10) to proceed by intermolecular transfer of phosphate between glucose 1,6-diphosphate and glucose 1-phosphate (or glucose 6-phosphate), yielding glucose 6-phosphate (or glucose 1-phosphate) while glucose diphosphate is regenerated.

The partially purified preparations of methylmalonyl CoA isomerase thus far available are contaminated with a methylmalonyl CoA decarboxylase and with succinyl CoA deacylase (2). Moreover, methylmalonyl CoA undergoes a slow, spontaneous decarboxylation to propionyl CoA and CO_2. For these reasons we have so far obtained only a partial dependence of the isomerization reaction on the presence of added propionyl CoA rather than the strict dependence expected from the transcarboxylation mechanism. Work is now in progress on the further purification of the enzyme.

Preparations—The preparation of partially purified isomerase from sheep kidney cortex (2), methylmalonyl CoA (2), methylmalonyl CoA labeled with C^{14} in the methyl group (2), and propionyl CoA-1-C^{14} (11) is described elsewhere. We are indebted to Dr. Martin Flavin for samples of propionyl CoA-1-C^{14}. Succinyl CoA and propionyl CoA were prepared from succinic and propionic anhydrides by the method of Simon and Shemin (12). Methylmalonyl CoA labeled with C^{14} in the carboxyl group was prepared by enzymatic carboxylation of propionyl CoA, in the presence of $C^{14}O_2$, as follows: A reaction mixture containing imidazole buffer, pH 7.0 (200 μmoles), reduced glutathione (10 μmoles), $MgCl_2$ (8 μmoles), ATP (8 μmoles), propionyl CoA (3 μmoles), $KHC^{14}O_3$ (20 μmoles, 6.2×10^5 c.p.m.), and pig heart propionyl CoA carboxylase (11) (specific activity 0.5, 2.3 mg. of protein), in a final volume of 1.0 ml., was incubated for 30 minutes at 30°. The mixture was then acidified with 0.2 ml. of 4.0 N HCl and heated for 20 minutes over a steam bath to remove $C^{14}O_2$,

neutralized with KOH, and centrifuged. The supernatant solution was used without further purification.

SUMMARY

1. Previous evidence that the immediate product of isomerization of methylmalonyl CoA is succinyl CoA has been strengthened by enzymatic identification of the latter.

2. In relation to the fact that methylmalonyl CoA has an asymmetric carbon atom, it has been found that only half of the chemically synthesized compound, but all of the methylmalonyl CoA synthesized by enzymatic carboxylation of propionyl CoA, can be isomerized by methylmalonyl CoA isomerase. Thus, as would be expected, enzymatic carboxylation of propionyl CoA yields the enantiomorph of methylmalonyl CoA which is attacked by the isomerase.

3. When the enzymatic isomerization methylmalonyl CoA $\rightleftharpoons$ succinyl CoA is conducted in the presence of C^{14}-labeled propionyl CoA, C^{14}-labeled succinyl CoA (or methylmalonyl CoA) is formed, and the specific radioactivities of the propionyl CoA and succinyl CoA are essentially the same. Labeled products are not obtained if $C^{14}O_2$ and unlabeled propionyl CoA are substituted for C^{14}-labeled propionyl CoA. This suggests that the isomerization occurs through a transcarboxylation, wherein the carboxyl group of methylmalonyl (or succinyl) CoA is transferred to an acceptor molecule of propionyl CoA, which is thereby converted to succinyl (or methylmalonyl) CoA, with release of 1 molecule of propionyl CoA.

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HYDROLYSIS OF I^{131}-THYROPROTEIN BY PANCREATIC ENZYMES*

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(Received for publication, November 21, 1957)

Until about 5 years ago, treatment with hot alkali was the most widely used method for hydrolyzing the iodoprotein of thyroid tissue. By such a procedure, Kendall first isolated thyroxine from the thyroid glands of oxen (1). Proteolytic enzymes have also been used for this purpose with varying degrees of success, since the early work of Hutchison (2), Oswald (3), and Harington (4). More recently, the usefulness of enzymic hydrolysis has been extended considerably by combining this method for fragmenting the thyroid protein with the very sensitive techniques of chromatography and radioisotopic labeling for the isolation and identification of the products of hydrolysis. Although this combination of procedures has been used in several laboratories (5–12), a thorough study of the hydrolysis with proteases has not been reported. In our investigation of this procedure we found, in agreement with Roche *et al.* (7), that the iodotyrosines are released quite rapidly from the thyroprotein, whereas the release of thyroxine proceeds more slowly. In addition, we found that the yield of thyroxine can be increased by performing the hydrolysis in the presence of certain metal cations (Mn^{++}, Mg^{++}, Ca^{++}). These findings are described below, along with a procedure for the analysis of I^{131}-labeled thyroid tissue. This procedure requires 10 to 20 hours of hydrolysis, yields approximately 95 per cent recovery of I^{131} protein in the form of I^{131}-amino acids, and results in little release of inorganic I^{131}.

EXPERIMENTAL

Source of Thyroid Tissue—I^{131}-labeled thyroid tissue was obtained from female rats (150 to 250 gm.) of the Long-Evans strain. Each animal had been injected, 8 hours to 9 days previously, with 50 to 100 μc. of carrier-free I^{131}-iodide. These rats were maintained on Purina laboratory chow containing 1 to 5 γ of iodine per gm.

Enzyme Digestion Procedure—The fresh tissue was homogenized with 15 volumes of ice-cold buffer solution in a motor-driven, all-glass tissue grinder. Insoluble material was removed by centrifugation for 10 minutes at 2800 r.p.m. (less than 5 per cent of the total I^{131} was lost with sediment).

* Aided by a grant from the United States Public Health Service.

300 μl. aliquots of the translucent, pink supernatant extract of thyroprotein were then mixed with 200 μl. portions of an enzyme solution consisting of 10 mg. of pancreatin suspended in buffer (higher concentrations of pancreatin do not improve the hydrolysis). Digestions were carried out in 15 × 127 mm. Pyrex test tubes kept at 37° in a constant temperature aluminum block tube heater. 1 drop of toluene was added to each sample

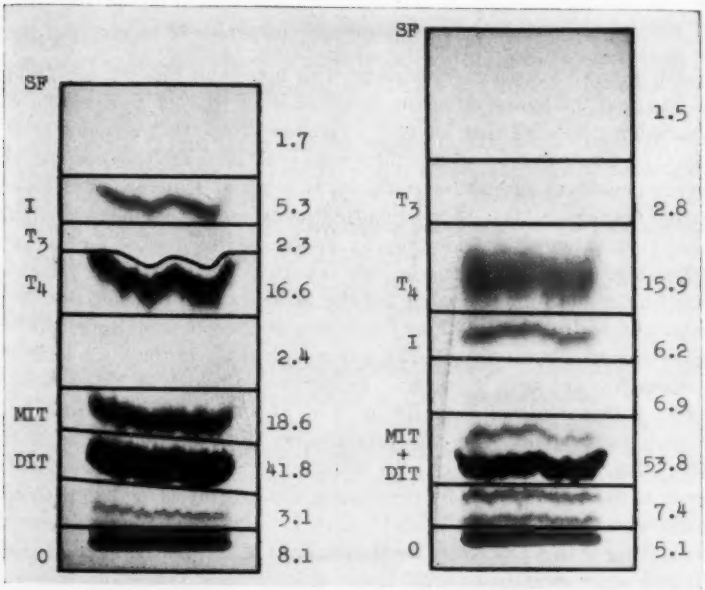

FIG. 1. 24 hour pancreatin digests of thyroid tissues taken from rats 68 hours after injection of 100 μc. of radioiodide tracer. Autographs of chromatograms developed in collidine-H_2O-NH_3 (left) and butanol-ethanol-2 N NH_4OH (right). Abbreviations on the left of each autograph identify the component present (see footnote 1), and figures on the right pertain to percentages of total I^{131} present on the chromatogram.

to prevent putrefaction, and 20 μl. of a 1 per cent solution of thiouracil were added to prevent further chemical changes from spurious oxidation of iodide. Metal ions, when present, were added as 10 μl. of 0.1 M solutions of their chloride or sulfate salts. Digestion was allowed to proceed for periods of 10 minutes to 120 hours.

Buffer Solutions—To determine what effect buffer composition or pH might have on the hydrolytic reaction, five different buffer mixtures were used for tissue digests: Krebs-Ringer bicarbonate; 0.11 M NaCl-0.04 M

$NaHCO_3$; 0.11 M NaCl-0.04 M Tris[1]; 0.11 M NaCl-0.04 M $Na_4P_2O_7$; and 0.05 M NH_4OH-0.05 M NH_4Cl. Glass-distilled water was used for all solutions.

Pancreatin Preparations—Five different pancreatin preparations were compared for effectiveness in the hydrolysis of I^{131}-thyroprotein and the release of I^{131}-thyroxine. Two were Merck U. S. P. preparations, one was a 3× U. S. P. preparation obtained from the Nutritional Biochemicals Corporation, and two were 4× U. S. P. preparations of the VioBin Corporation. The VioBin material was definitely superior, and was used exclusively in the present studies.

Chromatographic Analysis—20 μl. portions of tissue digests were subjected directly, *i.e.* without butanol extraction, to one-dimensional filter paper chromatographic analysis. The chromatographic and I^{131} assay procedures were essentially the same as those described previously (10–13). Most of the chromatograms were developed either with collidine-water (100:35) in an ammonia atmosphere or with a mixture of 10 parts *n*-butanol, 2 parts ethanol, and 4 parts 2 N NH_4OH (BEA). In Fig. 1, the radioautographs of two typical chromatograms of hydrolyzed thyroid tissue are reproduced to show how chromatograms were cut up for I^{131} assay.

The I^{131} contents of the various components are expressed as percentages of the total I^{131} found on each chromatogram. In Tables I to IV, the I^{131} values for each chromatogram total slightly less than 100 per cent, because the values for the I^{131} found in the areas between the MIT and thyroxine bands, and between iodide and the solvent front, are omitted.

Results

Effects of pH and Buffer Composition on Hydrolysis—The data in Table I were obtained from experiments designed to determine the pH and buffering conditions required for optimal hydrolysis of I^{131} protein and for optimal recovery of hydrolysis products. They indicate that, within the pH range of 8 to 9 and in the presence of added manganous ion, various buffer mixtures can be equally effective. In the case of pyrophosphate, however, there was a depression of both the hydrolysis of I^{131} protein and the release of I^{131}-thyroxine. This depression is probably related to the low solubility of the pyrophosphate salts of the alkaline earth metals and manganese.

Effects of Added Metal Cations—With the addition of certain metal cations (Mn^{++}, Mg^{++}, and Ca^{++}) to thyroprotein specimens before enzymic

[1] The following abbreviations are used: O, origin; DIT, diiodotyrosine; MIT, monoiodotyrosine; T_4, thyroxine; T_3, 3′,3,5-triiodothyronine; I, inorganic iodide; SF, solvent front; Tris, tris(hydroxymethyl)aminomethane; and BEA, butanol-ethanol-ammonia.

digestion, an enhancement of hydrolysis was observed. This metal ion effect appeared as a reduction in the I^{131} at the origin (Table II) and was accompanied by small but consistent increases in the yield of I^{131}-thyroxine.

TABLE I

Effect of pH and Buffer Composition on Pancreatin Hydrolysis of I^{131}-Thyroprotein

40 mg. per ml. of fresh thyroid tissue digested for 24 hours at 37° with 2 per cent VioBin 4× U. S. P. pancreatin. I^{131} composition of digest determined by paper chromatography with collidine-water solvent mixture.

Experiment No.	Buffer composition	pH of digest		Concentration of added Mn^{++}	Per cent total I^{131} recovered as					
		Initial	Final		Origin	DIT	MIT	T_4	T_3	I^-
				M						
1	Krebs-Ringer bicarbonate	8.1		0.004	7.1	48.5	19.6	16.9	2.9	5.7
2	0.11 M NaCl-0.04 M $NaHCO_3$	7.5	8.3	0.001	10.3	45.0	19.1	15.8	1.9	5.0
		8.1	8.7	0.001	13.0	43.7	18.1	16.7	1.8	4.4
		8.5	8.8	0.001	13.4	43.8	17.5	16.8	2.0	4.2
		9.1	9.0	0.001	17.1	42.0	17.3	15.0	2.0	4.3
		9.5	9.1	0.001	22.6	37.7	18.0	13.4	1.6	4.3
3	0.11 M NaCl-0.04 M $NaHCO_3$	7.4	8.8	0.001	14.5	41.8	15.3	15.3	2.7	5.9
		8.1	8.3	0.001	14.3	42.4	15.3	15.3	2.6	6.1
		8.6	8.8	0.001	16.5	41.7	13.7	14.2	2.7	5.6
	0.11 M NaCl-0.04 M $Na_4P_2O_7$	7.4	7.4	0.001	24.1	39.4	17.3	4.9	1.6	7.3
		8.1	8.4	0.001	29.0	33.5	16.5	5.3	1.0	6.5
		8.6	8.4	0.001	29.5	30.6	15.9	6.0	1.1	6.0
4	0.11 M NaCl-0.04 M $NaHCO_3$	7.9		0.002	10.3	44.8	19.4	16.4	2.4	5.1
	0.11 M NaCl-0.04 M Tris	7.6	7.7	0.002	7.1	47.2	21.2	13.8	2.2	6.1
		8.1	8.0	0.002	6.8	45.7	20.6	16.7	2.5	5.3
		8.6	8.5	0.002	9.2	45.4	19.6	16.0	2.1	5.9
		9.3	9.0	0.002	14.4	42.7	18.2	15.0	1.9	5.6
5	Krebs-Ringer bicarbonate	8.0		0.004	5.3	38.6	25.4	23.7	3.2	3.9
	0.11 M NaCl-0.04 M Tris	8.3		0.004	4.7	39.0	26.0	23.2	3.8	3.4
6	0.11 M NaCl-0.04 M Tris	8.5		0.004	5.0	43.5	25.7	16.7	2.5	4.9
	0.05 M NH_4OH-0.05 M NH_4Cl	9.0		0.004	6.2	44.0	23.8	15.0	2.8	5.3
7	0.05 M NH_4OH-0.05 M NH_4Cl	8.6		0.005	4.2	43.2	25.8	16.6	2.7	3.4

The curves of Fig. 2 show augmentation not only of the final yield of I^{131}-thyroxine, but also of the rate at which the I^{131}-thyroxine was released.

TABLE II

Effect of Added Metal Cations on Hydrolysis of Thyroprotein with Pancreatin

Rat thyroid tissue taken 24 or 48 hours after I^{131} injection and hydrolyzed for 16 to 24 hours with VioBin 4× U. S. P. pancreatin in either saline-bicarbonate or saline-Tris buffer.

Experiment No.	Cation added	Concentration of cation added	Per cent hydrolysate I^{131} found as					
			Origin	DIT	MIT	T_4	T_3	I^-
		M						
8	None		8.8	45.4	23.5	11.3	2.3	5.7
	Mn^{++}	0.001	5.6	45.2	24.3	15.6	3.2	4.4
	"	0.002	5.2	46.4	23.2	16.8	2.9	4.0
	"	0.005	4.1	46.9	22.4	20.0	2.8	2.7
	"	0.01	4.5	48.1	22.3	19.1	2.7	2.4
9	None		19.1	43.1	18.4	12.3	2.1	4.5
	Mn^{++}	0.002	16.2	43.3	18.2	15.4	2.2	3.7
	"	0.001	15.1	43.1	18.5	16.0	2.3	3.7
	"	0.002	10.7	44.5	18.9	18.6	2.5	3.6
	"	0.01	10.9	46.4	18.4	17.6	2.4	3.4
10	None		14.4	46.1	21.0	11.0	1.5	5.6
	"		13.0	47.6	20.8	11.5	1.8	5.6
	Mn^{++}	0.002	10.7	47.3	20.7	14.8	1.7	5.1
	"	0.002	9.7	46.5	21.3	15.7	2.0	4.8
	Mg^{++}	0.003	10.6	47.6	21.6	13.0	1.5	6.0
	"	0.003	10.4	48.1	21.7	12.4	1.7	5.7
	Ca^{++}	0.002	11.6	45.1	22.4	14.2	1.7	5.1
	"	0.002	9.3	48.7	20.7	14.1	2.3	5.0
	Co^{++}	0.002	17.5	44.4	21.2	8.1	2.1	6.8
	"	0.002	15.7	44.8	22.3	7.9	2.4	6.6
11	None		15.4	43.4	18.8	15.4	2.6	4.9
	"		15.6	44.0	18.2	14.6	2.4	5.4
	Mn^{++}	0.002	11.4	43.7	18.9	18.9	3.0	4.2
	Mg^{++}	0.002	14.1	44.7	18.4	15.7	2.9	4.5
	Ca^{++}	0.002	12.7	43.5	18.9	16.9	3.2	4.7
	Ba^{++}	0.002	12.4	45.0	18.6	15.7	2.9	5.4
	Cd^{++}	0.002	25.6	41.6	17.4	9.8	2.3	3.3
	Ni^{++}	0.002	15.8	44.1	18.4	13.9	2.8	5.1

This finding is of particular interest to those studying the process of thyroxine biosynthesis, since other workers have reported that the hydrolytic release of thyroxine from thyroprotein is especially difficult (14). Roche

and his coworkers frequently used hydrolysis with papain after treatment with pancreatin in order to obtain adequate yields of the iodothyronines. In the present experiments, hydrolysis in the presence of 0.005 M $MnSO_4$ was found to increase thyroxine yields by 25 to 80 per cent.

It is interesting to note that the added cations influenced specifically the recovery of I^{131}-thyroxine. The yields of MIT and DIT (Table II) were not significantly altered by the added Mn^{++}, Mg^{++}, or Ca^{++}.

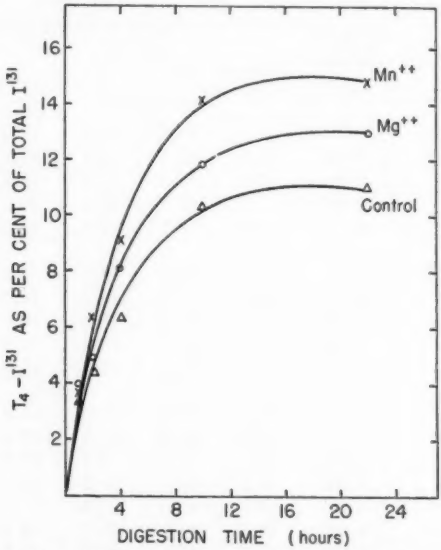

FIG. 2. Effect of added 0.005 M $MnSO_4$ and $MgSO_4$ on the release of I^{131}-thyroxine (T_4-I^{131}) from labeled rat thyroprotein during incubation with 2 per cent pancreatin in saline-bicarbonate buffer. Each curve was obtained from I^{131}-thyroxine values determined on aliquots withdrawn from a single hydrolysate at various times during the incubation.

Rate of Hydrolysis—By chromatographing aliquots of thyroid digests at various stages of hydrolysis, the rates of disappearance of iodoprotein and appearance of iodoamino acids were measured. Some of these data are shown in Table III. These findings indicate that the proteolytic release of the iodotyrosine, especially MIT, is rapid. Within the 1st hour of digestion, a maximal value for I^{131}-MIT was obtained which was not altered greatly by continued digestion. This maximal value presumably represented complete release of I^{131}-MIT from the thyroprotein. Within the first 10 minutes of digestion, the I^{131}-MIT frequently amounted to more than 70 per cent of the maximal value. Maximal release of I^{131}-DIT

required approximately 10 hours under the same conditions. The rate of appearance of thyroxine was variable, but definitely slower than that of the iodotyrosines. Since the recovery of thyroxine is often of considerable interest, the rate of appearance of I^{131}-thyroxine is plotted in Fig. 3

TABLE III

Rate of Hydrolysis of I^{131}-Thyroprotein by Pancreatin

Rat thyroid tissue taken 24 or 48 hours after I^{131} injection and digested at 37° with 2 per cent VioBin 4× U. S. P. pancreatin in saline-Tris buffer with 0.004 M $MnSO_4$.

Experiment No.	Digestion time	Per cent total I^{131} found as					
		Origin*	DIT	MIT	T_4	T_3	I^-
	hrs.						
1	4	13.6	46.2	19.0	14.9	2.7	3.5
	8	9.4	47.1	19.5	18.3	2.6	3.3
	12	8.0	48.3	19.8	17.0	3.0	4.0
	18	7.7	46.0	20.6	17.1	3.1	5.2
	25	7.1	48.5	19.6	16.9	2.9	5.7
	min.						
2	10	42.6	26.4	16.9	9.2†		4.8
	20	31.3	34.1	21.5	9.1		3.8
	30	29.0	37.5	20.6	9.6		3.3
	hrs.						
	1	27.1	39.2	21.6	6.8	1.9	3.6
	3	17.8	41.5	21.8	12.2	1.8	5.2
	6	11.0	43.9	23.7	14.5	2.1	4.9
	11	7.5	44.4	24.7	16.4	2.3	4.7
	24	5.8	46.4	23.2	15.0	2.4	5.6
3	0.5	29.3	35.6	23.7	3.8		2.4
	1	24.3	38.0	24.4	5.5	1.5	3.1
	3	16.3	42.1	24.2	10.3	2.6	3.4
	6	9.8	43.2	24.8	16.4	2.6	2.4
	11	6.6	44.5	24.6	18.5	3.5	2.3
	23	4.8	44.0	26.0	14.5	1.5	6.8

* Includes the region between origin and DIT.

† T_4 and T_3 sections assayed together.

for four representative experiments. Maximal yields of I^{131}-thyroxine were obtained after periods of digestion ranging from 8 to 24 hours. Furthermore, with prolonged digestion, thyroxine recovery tended to decline and, after 3 days of digestion, was, in one case, 65 per cent of the maximal value. Concurrent with the loss of I^{131}-thyroxine, increasing radioactivity appeared in the inorganic iodide component (Table III). These findings indicate that significant decomposition of thyroxine can occur with prolonged hydrolysis.

Nature of I^{131} in "Origin" Components

Since unhydrolyzed protein is known to remain at the origin during chromatography, the I^{131} present in the "origin" components reflects the incompleteness of the hydrolysis. Values found for this component normally ranged from 4 to 10 per cent of total I^{131} after 24 hours of digestion with pancreatin (Table IV), thereby indicating that hydrolysis by this procedure was more than 90 per cent complete with regard to release of I^{131}-labeled amino acids.

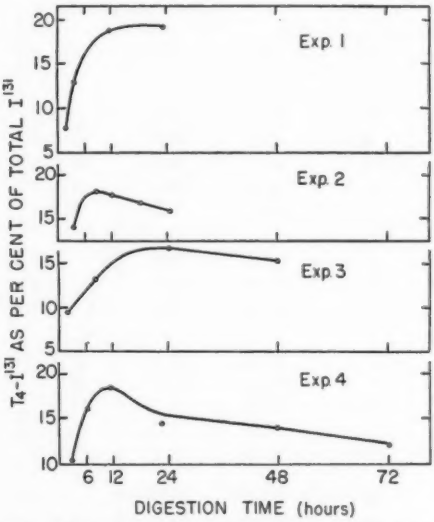

FIG. 3. Comparison of the rates of I^{131}-thyroxine (T_4-I^{131}) release during pancreatic digestion of four different rat thyroid specimens labeled *in vivo* with I^{131}.

Further study on the nature of I^{131} in the origin component revealed that, by chromatographing digests in a more aqueous BEA mixture,[2] the origin material could be separated into at least four new components which are probably polypeptides, since they appeared early in the digestion and diminished in intensity as the individual amino acids were released. After only 10 or 20 minutes of digestion, approximately 40 per cent of the total I^{131} was found among these four polypeptides, and about 45 per cent in MIT and DIT, whereas only 1 to 3 per cent remained at the origin as unchanged thyroglobulin. Apparently, thyroglobulin is rapidly split into these polypeptide fragments, which are then hydrolyzed more slowly to release the iodotyrosines and iodothyronines.

[2] 10 parts *n*-butanol, 4 parts ethanol, 8 parts 2 N NH_4OH.

I^{131} of Thyroid Glands at Various Times after I^{131} Administration—As shown in Table IV, the I^{131} composition of the thyroid glands of the rats from our colony was constant over a period of 8 hours to 9 days after a single injection of carrier-free I^{131}. Values for I^{131}-DIT remained between

TABLE IV

Chromatographic Fractionation of Rat Thyroid Iodine at Various Times after Injection of I^{131}

Thyroid tissues hydrolyzed for 24 hours in 0.11 M NaCl-0.05 M Tris at pH 8.5 with 2 per cent VioBin 4× U. S. P. pancreatin and 0.004 M $MnSO_4$. Each analysis was performed on duplicate aliquots taken from a homogenate of thyroid tissue pooled from four rats.

Time after I^{131} injection	Per cent hydrolysate I^{131} found as					
	Origin*	DIT	MIT	T_4	T_3	I^-
hrs.						
8	4.7	46.5	26.8	14.5	2.1	4.2
	4.5	45.6	26.7	15.2	2.4	4.2
26	5.8	48.2	22.3	15.5	2.3	4.2
days						
2	6.6	45.5	19.6	19.9	2.6	3.9
	6.0	46.4	19.9	19.2	2.6	4.1
3	6.9	42.8	20.6	20.6	3.1	4.0
	5.9	46.8	18.9	20.5	2.7	3.6
4	6.3	45.3	18.9	21.6	2.8	3.6
	6.5	46.4	18.4	20.6	2.2	4.1
7	7.7	45.7	16.6	23.3	2.8	3.9
	7.5	45.3	16.5	23.9	2.7	3.9
9	8.2	47.3	19.0	19.3	2.4	3.9
	8.7	46.3	18.5	20.4	2.1	3.8

* Includes the section between the origin and DIT.

43 and 48 per cent during this period, those for I^{131}-MIT decreased from 27 to 19 per cent, and for I^{131}-thyroxine they increased from 15 to 20 per cent; for I^{131}-T_3 they did not vary significantly from values of 2 to 3 per cent of total I^{131} of thyroid glands. The identities of the various I^{131}-containing amino acids were determined by cochromatography with added pure compounds. However, since the iodothyronines are not well defined by chromatography with the collidine-solvent mixture, the presence of small

amounts of 3,3′-diiodothyronine and 3,3′,5′-triiodothyronine, as reported by Roche *et al.* (15, 16), cannot be excluded.

In the same thyroid glands, the inorganic I^{131}-iodide content was found to be between 3.81 and 4.23 per cent of total I^{131}. Such values are low and not significantly different from the I^{131}-iodide content of the unhydrolyzed tissue. It was therefore concluded that little deiodination of organically bound I^{131} occurred during the analytical procedures.

DISCUSSION

The enzymic hydrolytic technique was discarded long ago in favor of alkaline hydrolysis because it seemed to be lacking in completeness, and because it was accompanied by the release of large amounts of inorganic iodide (3, 4), an indication that considerable deiodination of organic iodine was occurring. With the application of newer methods of analysis (*e.g.* I^{131} and chromatography), and with the refinements of the enzymic technique described above, these objections seem no longer to be valid. In a previous report (13), we described an analytical procedure for thyroidal iodine which involved hydrolysis of the tissue for 16 hours in hot 2 N NaOH. During this hydrolysis, 13 to 21 per cent of the I^{131} protein was released in inorganic form. In comparison, a 24 hour digestion with the VioBin pancreatin gave 95 per cent complete hydrolysis of I^{131} protein with almost negligible release of inorganic iodide.

Further advantages of the enzymic digestion procedure over the alkaline hydrolytic method might also be mentioned here. The tedious acid butanol extraction and solvent evaporation steps are eliminated so that all of the I^{131} in a tissue specimen can be accounted for on a single chromatogram of a pancreatin hydrolysate. The elimination of the butanol extraction and alkaline hydrolytic procedures also helps to reduce the possibility of the artifactual formation of labeled iodine compounds which arise through exchange reactions under the acid conditions of butanol extraction and evaporation, or the degradative and condensation reactions during heating with strong alkali (17–20). In order to make certain that new sources of artifactual labeling of iodine-containing compounds were not introduced with the enzymic digestion procedure, test analyses were performed on non-radioactive thyroid specimens to which purified radioiodide was added *in vitro*. Chromatograms of such thyroid hydrolysates consistently showed only a single band for the radioiodide.

The mechanism by which the metal cations enhance the yield of thyroxine during digestion is not entirely clear. The fact that increased I^{131}-thyroxine yield is accompanied by decreased I^{131} recovered in the origin component suggests that the metal ions act, in part at least, by activating the metal peptidases present in the pancreatin so that a more complete

hydrolysis is obtained. On the other hand, since it is known that thyroxine is somewhat unstable, and hence that some decomposition may be expected to occur during the analysis, it is conceivable that the metal ions might stabilize the thyroxine by forming some sort of complex with it. These considerations call to mind the observations of Reineke (21) concerning the catalytic action of manganese oxides on the formation of thyroxine during the iodination of casein and the oxidation of diiodotyrosine. At that time, it was suggested that the manganese served as an oxygen carrier for the oxidative coupling of the iodotyrosine. However, it is unlikely that such a mechanism was functioning in the present experiments since all of the analyses were carried out in the presence of excess reducing agent (thiouracil).

SUMMARY

1. A procedure is described for the analysis of I^{131}-containing compounds in labeled thyroid tissue. This procedure makes use of an enzymic hydrolysis technique that gives approximately 95 per cent recovery of the I^{131}-thyroglobulin in the form of I^{131}-amino acids.

2. The yield of thyroxine obtained from thyroprotein is augmented by hydrolysis in the presence of Mn^{++}, Mg^{++}, or Ca^{++}.

3. It was found that during the enzymic digestion maximal release of monoiodotyrosine occurred within 1 hour and that release of diiodotyrosine required less than 10 hours, whereas maximal thyroxine release required 8 to 24 hours. Digestion beyond 24 hours usually resulted in decreased yields of thyroxine.

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STUDIES ON THE BIOLOGICAL FORMATION OF GLUCOSAMINE IN VIVO*

I. ORIGIN OF THE CARBON CHAIN

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(Received for publication, January 28, 1958)

It was not until a few years ago that knowledge concerning the biological formation of D-glucosamine was obtained. Since that time it has been shown by techniques *in vivo* that, in the rat (1) and bacteria (2), the carbon chain of glucosamine is formed intact from glucose. A previous communication from this laboratory (3) has shown that this conversion also takes place in the bird and probably represents the major pathway of glucosamine formation. The following report presents the details of this work.

Methods

Conditioning of Animals—Hens of the New Hampshire Red variety, which weighed 2 kilos and were 5 to 7 months old, were kept in standard hen metabolism cages. These cages were covered with an air-tight plastic covering fitted with an intake tube attached to a soda lime tower at the bottom of one side of the cage and with an outlet tube at the top of the opposite side to permit the collection of the respiratory carbon dioxide. The hens were fed a standard laying mash *ad libitum* and, in addition, were given 40 gm. of scratch feed plus some cracked oyster shell each night. When the hen had attained a regular egg laying cycle, it was fed some non-radioactive glucose contained in a gelatin capsule *per os* every 4 hours for a period of 3 days. If the animal was able to withstand this treatment and still maintained its egg laying schedule, which was one egg every day for 3 consecutive days and then no egg the following day, it was considered ready for the feeding of the isotopic compound. 4.5 mg. of glucose-1-C^{14} contained in a gelatin capsule (specific activity, 22.5 μc.

* Aided by grants from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and the Damon Runyon Memorial Fund for Cancer Research, Inc.

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per mmole) were then given *per os* early in the morning of the 1st rest day (4) and then every 4 hours for 3 days. The first egg was then laid the following morning, and 28 hours later the second egg was laid. 10 hours after that time the animal was sacrificed, and the third egg was removed from the uterus before any shell formation had occurred. Prior to removal of the third egg the animal was anesthetized with ether; the blood was drained from the jugular vein and defibrinated, and the liver was removed.

Isolation of Glucosamine—The ovomucoid of the egg white was used as the source of the glucosamine. After the egg white was separated from the yolk of the freshly laid egg the albumins and globulins were coagulated by heating at pH 5 and separated from the mother liquor. The mother liquor was then filtered through a layer of Celite analytical Filter-Aid (Johns Manville), concentrated, and mixed with 4 to 5 volumes of ethyl alcohol to precipitate the crude ovomucoid. This ovomucoid, after being dried, was then heated in 1.2 N HCl at 100° (5), and a maximal yield of glucosamine as determined colorimetrically (6) was obtained in 4 hours. The hydrolysate was then treated with a 15 per cent solution of phosphotungstic acid in 0.1 N HCl to precipitate the protein and basic amino acids and centrifuged. The supernatant solution was cooled at 4° for 24 hours and filtered. The resulting clear filtrate was then extracted three times with redistilled isoamyl alcohol containing 10 per cent ethyl ether to remove the excess phosphotungstic acid and evaporated to dryness *in vacuo* at a bath temperature of 40°.

An additional hydrolysis in acid was found necessary to effect a good yield of the α-naphthylidene derivative which was used for radioactive counting. This hydrolysis probably deacetylated any *N*-acetylglucosamine and also served to split any complexes of glucosamine and phosphotungstic acid. To the residue remaining after distillation were added 20 ml. of 20 per cent HCl, and the solution was heated at 100° for 2 hours. No glucosamine was lost by this heating. The hydrolysate was decolorized with Norit and filtered, and the solution was again evaporated *in vacuo* to a thick syrup to remove the excess HCl. Since the solution at this point still contained contaminating amino acids, further purification of the glucosamine was obtained by ion exchange chromatography.

Dowex 1 resin (20 to 40 mesh), which had previously been converted to the basic form with 1 N NaOH, then washed with 1 N Na_2CO_3, and again treated with 1 N NaOH was used in a column of 1 cm. diameter filled approximately to a height of 25 cm. The treatment with sodium carbonate appeared to remove an impurity from the resin which eluted with the glucosamine and interfered with the formation of the derivative. In order that additional cations would not be added to the influent solution,

the basic properties of the resin were used to neutralize the excess acidity and to convert the amino acids to their anionic form. The column was operated initially at 4° to minimize the destruction of the glucosamine in the basic solution produced by the splitting of neutral salts by the resin and also to slow down the rate of passage of the glucosamine through the resin. The cold solution of glucosamine in 40 ml. of water was passed through the column at a flow rate of 0.5 ml. per minute until 40 ml. of effluent were obtained. The pH of the effluent at this point was 10. Cold water was then passed through until the pH dropped to about 7, at which point the glucosamine started to emerge. The column was then

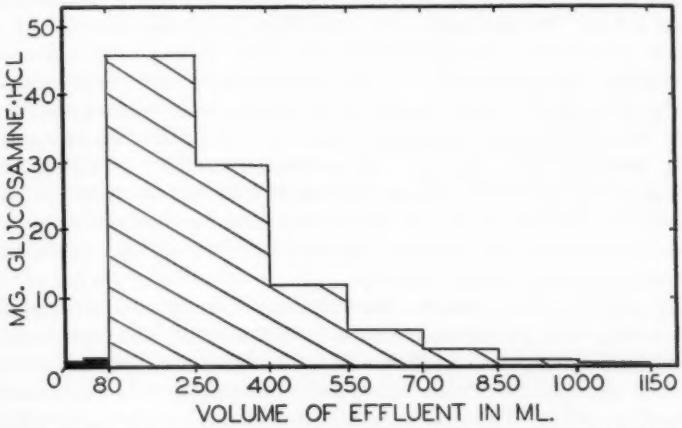

FIG. 1. Elution of glucosamine from Dowex 1 (OH^-) (20 to 40 mesh) by water. The first 80 ml. were eluted at 4° and the remainder at room temperature.

warmed to room temperature and all further elution with water was done at a flow rate of 3 ml. per minute; 200 ml. fractions were collected. The use of the resin at two temperatures permitted the separation of the glucosamine from the cations present in the influent solution. A typical elution curve is seen in Fig. 1.

The fractions containing the glucosamine were concentrated *in vacuo* to a final volume of about 3 ml. The purity of the concentrate, at this point, was determined by ascending paper chromatography on Whatman No. 1 paper with the use of three solvent systems: phenol saturated with water plus 1 per cent ammonia (7), phenol saturated with water, and 80 per cent *n*-propanol containing 0.8 per cent ammonium acetate (8). The over-all yield of glucosamine as determined by colorimetric analysis was 68 to 85 per cent.

The glucosamine hydrochloride was precipitated by adding acetone to

the concentrated eluate and by chilling the solution overnight at 4°. The resulting precipitate was separated by centrifugation and dried *in vacuo* over calcium chloride. The glucosamine hydrochloride was further purified by conversion to its 2-hydroxy-α-naphthylidene derivative by the method of Jolles and Morgan (9). Crystallization of the derivative by their method was found unsatisfactory, and the following procedure was followed. The dried crude Schiff base was dissolved in a minimal amount of absolute methanol and centrifuged to remove any insoluble residue. This methanolic solution was then slowly evaporated by use of a stream of dry nitrogen with periodic additions of small amounts of acetone to reduce the solubility of the derivative. When the volume was reduced to about 0.5 ml., the precipitate was centrifuged, and the supernatant solution was removed. The precipitate was then washed with cold acetone and finally with cold water. It was then dried *in vacuo*, and crystallization was repeated. Final drying in all cases was *in vacuo* over P_2O_5 at 78°. The melting point of the derivative was 195.5–196.5° (decomposition) when measured in a heating bath preheated to 180°. Analysis of the derivative was as follows: found, C 61.05, H 5.72, N 4.18; theory, C 61.25, H 5.75, N 4.20 per cent. All derivatives used for radioactive counting were recrystallized to constant specific activity and characterized by melting point and nitrogen analysis.

Degradation of Glucosamine—The radioactive 2-hydroxy-α-naphthylidene glucosamine was decomposed with 4 N HCl at 100°, and carrier glucosamine hydrochloride was added. The 2-hydroxy-α-naphthaldehyde was removed by extraction with ether, and the solution was evaporated *in vacuo* to a syrup. The glucosamine hydrochloride was precipitated with acetone, collected by centrifugation, and dried. The recovery was 96 per cent. The glucosamine was then oxidized to glucosaminic acid according to the procedure of Pringsheim and Ruschmann (10) except that, after evaporation *in vacuo*, the glucosaminic acid was precipitated as suggested by Wolfrom and Cron (11). The acid was recrystallized twice and dried *in vacuo*. The identity of the product was confirmed by use of ascending chromatography on Whatman No. 1 paper with the propanol-acetate solvent. The R_F of glucosaminic acid in this solvent is 0.09.

The glucosaminic acid was decarboxylated by the use of chloramine-T (12) in an aeration apparatus. The carbon dioxide formed was aerated over and collected in the usual way. The $BaCO_3$ was plated from an acetone suspension on an aluminum planchet for radioactive counting. Quantitative yields were obtained.

Isolation of Blood Glucose and Glycogen Glucose—The proteins in the defibrinated blood were removed with zinc sulfate and barium hydroxide according to the method of Somogyi (13). The filtrate was concentrated

in vacuo, and the glucose was converted to its phenylosazone according to the method of Feller *et al.* (14). The glucosazone was recrystallized to constant specific activity from 50 per cent ethanol.

The liver glycogen was isolated according to the method of Good *et al.* (15). After three reprecipitations, it was dried and hydrolyzed with 0.6 N HCl at 100° for 4 hours. The glucose formed was then converted to its phenylosazone.

The osazones were plated on an aluminum planchet from an ethereal suspension and the naphthylidene derivative of glucosamine from acetone. All samples were counted to a standard error of 5 per cent with an end window Geiger-Müller tube and corrected to infinite thinness.

RESULTS AND DISCUSSION

The feeding of the isotopic glucose-1-C^{14} at intervals of 4 hours was done in an attempt to stabilize the specific activity of the blood glucose at a constant level. That stabilization was fairly well attained within 9 hours of the first feeding is shown in Table I. In this experiment the specific activities of the respiratory carbon dioxide collected at various times during the feeding period are recorded as an index of the constancy of the specific activities of the carbohydrate available for oxidative and synthetic purposes in the hen.

The specific activity of the glucosamine isolated from the eggs after feeding glucose-1-C^{14} increased until it equaled or slightly exceeded that of the blood glucose and glycogen glucose (Table II). Although the specific activity of the glucosamine from Egg 3 was somewhat high, the specific activities of the glucosamine from Eggs 2 and 3 approximate closely the specific activities of the blood and glycogen glucose isolated. These data would indicate that either glucose or some closely associated metabolic product (*i.e.* a phosphorylated hexose) is the direct precursor of glucosamine. Since the specific activity of the glycogen glucose is approximately the same as that of the blood glucose, it may be assumed that this glycogen glucose reflects the average specific activity of all the cellular hexoses of which one may be the direct precursor of glucosamine. The recent studies of Leloir and Cardini (16), in which enzymes from *Neurospora crassa* were used, indicate that a phosphorylated hexose actually is the precursor. The further work of Blumenthal *et al.* (17) has demonstrated that this phosphorylated hexose is indeed fructose 6-phosphate.

Since the glucose fed was labeled only in carbon 1, the ratio of the specific activity of carbon 1 to the average specific activity of the entire molecule should be 6 if the carbon chain of glucose was converted to glucosamine without any skeletal rearrangement of the chain. Partial degradation of

TABLE I

Specific Activity of Respiratory Carbon Dioxide after Feeding Glucose-1-C^{14} to Hen

Elapsed time after initial feeding	Specific activity of respiratory CO_2
hrs.	*c.p.m. per mmole*
2	1140
3	842
8	992
9	2720
10	2840
11	2890
28	2940
30	3010
32	2890
56	3130
58	2850

The specific activity of the glucose-1-C^{14} was 22.5 μc. per mmole. 4.5 mg. of glucose-1-C^{14} were fed every 4 hours for 3 days.

TABLE II

Incorporation of C^{14} into Glucose, Glycogen, and Glucosamine

Compound	Specific activity
	c.p.m. per mmole
Blood glucose	18,000
Liver glycogen	16,600
Glucosamine (Egg 1)	781
" (" 2)	16,000
" (" 3)	22,100

The blood glucose and liver glycogen were obtained by sacrificing the hen 10 hours after the second egg was laid. The glucose and glycogen were counted as their phenylosazones and the glucosamine as its 2-hydroxy-α-naphthylidene derivative. The radioactivity measurements were corrected to infinite thinness.

TABLE III

Specific Activities of Carbons of Glucosamine after Feeding Glucose-1-C^{14}

Sample	Specific activities of carbon atoms of glucosamine		
	Average of all 6 carbon atoms	Carbon atom 1	Specific activity of carbon 1 / Average specific activity of glucosamine carbons
	c.p.m. per mmole C	*c.p.m. per mmole C*	
Glucosamine (Egg 2)	2700	12,500	4.6
" (" 3)	3600	15,900	4.4

All samples were counted as $BaCO_3$, and the radioactivity measurements were corrected to infinite thinness.

the glucosamine from Eggs 2 and 3 (Table III) showed this ratio to be 4.6 and 4.4, respectively, and that 77 and 74 per cent of the total isotope was in carbon atom 1. Thus it appears that the major portion of the isotope was incorporated from glucose fed without any skeletal rearrangement of the carbon chain. The remainder of the radioactivity in the carbons other than carbon 1 may be assumed to have been derived from glucose in which the carbon atoms had been randomized via reactions concerned with the Embden-Meyerhof pathway.

These results are in good agreement with the work done with bacteria and the rat where the carbon chain of glucose was shown to be used directly for the synthesis of glucosamine. In addition, the data are in accord with the studies *in vitro* of Leloir and Cardini (16) and of Blumenthal *et al.* (17).

SUMMARY

A study has been made of the origin of the carbon chain of glucosamine *in vivo* in the chicken. A method has been described for the isolation of this amino sugar from the ovomucoid of the egg white. It has been shown that approximately 75 per cent of the isotope in the glucosamine isolated after feeding glucose-1-C^{14} was in carbon atom 1 and that the ratio of carbon 1 to the entire molecule was about 4.5.

These data indicate that the major part of the carbon chain of glucosamine isolated from the ovomucoid of the egg was derived from glucose without scission of the carbon chain.

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STUDIES ON THE BIOLOGICAL FORMATION OF GLUCOSAMINE IN VIVO*

II. ORIGIN OF THE NITROGEN ATOM

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(Received for publication, January 28, 1958)

Although the carbon precursor of glucosamine has been studied in many organisms (1–5), only recently has work been done on establishing the precursor of the nitrogen atom. A preliminary report from this laboratory (6) showed that ammonia was an excellent source of this nitrogen. Recent work by Leloir and Cardini (1) has shown that, in a crude enzyme system from *Neurospora crassa*, the amide group of L-glutamine was the precursor of the nitrogen of glucosamine. This same observation has been made in experiments with rat liver (7) and streptococci (8). Ammonia itself can also be the source of this nitrogen atom in the synthesis of glucosamine 6-phosphate by the glucosamine-6-phosphate deaminase system prepared from *Escherichia coli* (9) and from rat and pig liver (10).

In this paper are presented the details of the previously reported findings, and data are presented showing that ammonia nitrogen is a precursor of the nitrogen atom of glucosamine isolated from the ovomucoid of the chicken egg.

Methods and Materials

The methods employed in the conditioning of hens of the New Hampshire Red variety and the isolation of the glucosamine from ovomucoid have been described (5). Samples of nitrogen for mass analysis were prepared from the various compounds by the method of Rittenberg (11).

The radioactive samples in the case of the glycine experiment were counted with a thin end window Geiger-Müller tube or in a gas flow counter with a 99.05 per cent helium-0.95 per cent isobutane mixture as the quenching gas. All samples were counted to a standard error of 5 per cent.

* Aided by grants from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and the Damon Runyon Memorial Fund for Cancer Research, Inc.

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Uric acid was isolated according to the procedure of St. John and Johnson (12) and was characterized by its nitrogen content before degradation for mass analysis by one of three separate methods. In the first method glycine was obtained by hydrolysis of uric acid in concentrated HCl at 150° (13) and was isolated as the benzoyl derivative. The α-carbon and nitrogen atom of glycine are derived from C_5 and N_7 of uric acid. The carboxyl carbon, however, is derived equally from C_4 and C_6 (14). In the second method uric acid was oxidized with chlorine to give nitrogen atoms 7 and 9 as urea and alloxan (15, 16). This latter compound was converted to alloxantin which was isolated and subsequently oxidized with PbO_2 to urea. This urea consists of nitrogen atoms derived from N_1 and N_3 of uric acid. Finally uric acid was oxidized by the alkaline peroxide method of Brandenberger (17) to yield oxonic acid and ammonia which is derived equally from N_1 and N_7.

N^{15}-Glycine was synthesized from N^{15}-potassium phthalimide (Eastman Organic Chemicals) according to the procedure of Schoenheimer and Ratner (18). It was then mixed with 0.5 mc. of glycine-1-C^{14} (Tracerlab), dissolved in water, and recrystallized by adding 5 volumes of warm ethanol and by cooling to 0°. The product had a N^{15} concentration of 30.4 atom per cent excess and a specific activity of 0.78 μc. per mmole (end window counter). The C^{14}:N^{15} ratio was 56,700 c.p.m. per mmole per atom per cent excess N^{15}.

In each experiment the isotopic compound contained in a gelatin capsule was fed to a chicken weighing approximately 2 kilos. The animal had previously been conditioned to lay an egg on 3 consecutive days and then rest a day. In the experiment with $N^{15}H_4Cl$, 200 mg. were fed every 8 hours for a total of nine feedings. 242 mg. of N^{15}-glycine-1-C^{14} were fed in the same way. No untoward effects on the animals were noted when the above amounts of NH_4Cl or glycine were fed over the same period of time in a trial experiment. Fecal droppings were collected over a 12 hour period, and the pooled droppings were used for the isolation of uric acid.

RESULTS AND DISCUSSION

Feeding of $N^{15}H_4Cl$—It has been shown that ammonia N is readily incorporated into uric acid (19, 20). More recent work by Sonne *et al.* (16), Shemin and Rittenberg (21), and Levenberg *et al.* (22) has established the precursors of each of these nitrogen atoms. This work has demonstrated that N_1 of uric acid is derived from aspartic acid, N_3 and N_9 from the amide nitrogen of glutamine, and N_7 from the glycine nitrogen. Therefore the uric acid excreted was used as a comparative index of the efficiency of the incorporation of $N^{15}H_4Cl$ into glucosamine.

Eggs were laid by the hen 26, 54, and 81 hours after the initial feeding

of $N^{15}H_4Cl$. After missing the next day two more eggs were laid. This latter cycle was repeated; a total of seven eggs from which glucosamine was isolated and analyzed for N^{15} concentration was obtained. The results of the experiment are shown in Fig. 1 where average N^{15} values of the uric acid are compared to the values for the N^{15} concentration of the glucosamine. The values for glucosamine are plotted for the estimated time of egg white deposition about the yolk of the egg. This egg white deposition, which occurs 3 to 4 hours after ovulation (23), is followed rapidly by the formation of a shell membrane, thus rendering the egg white metabolically inert with respect to the rest of the body. The shell is formed during the remainder of the time before the egg is laid. Since the dura-

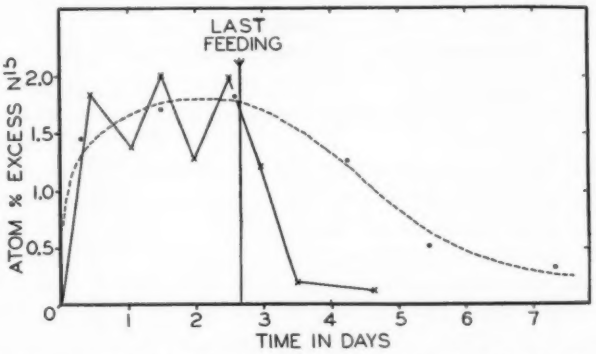

FIG. 1. Incorporation of N^{15} into uric acid and glucosamine after feeding $N^{15}H_4Cl$ to a hen. ×, the uric acid; ○, glucosamine. The N^{15} concentration of the glucosamine is plotted at an approximated time of egg white formation.

tion of the laying cycle was about 26 hours, an arbitrary value of 18 hours was subtracted from the time the egg was laid to approximate the time of egg white formation. The N^{15} concentration of the glucosamine at this time is then an average measure of the N^{15} incorporated during the preceding period. The use of this estimated time demonstrates better the relationship of the N^{15} concentration of the glucosamine to the N^{15} concentration of the uric acid as a function of time.

The curve shows that the incorporation of N^{15} into the glucosamine from fed $N^{15}H_4Cl$ is very rapid and occurs at roughly the same rate as N^{15} incorporation into uric acid. It would appear, therefore, that both the glucosamine and uric acid have a nitrogen precursor in common.

The jagged appearance of the uric acid curve can be attributed to the fact that two isotopic feedings occurred during one 12 hour period in which droppings were collected, while only one feeding occurred during the next

collection period. This double feeding would tend to keep the N^{15} concentration of the uric acid precursors higher. At the cessation of feeding $N^{15}H_4Cl$ the N^{15} concentration of the uric acid dropped off precipitously, evidence indicating that the N^{15} concentration of the uric acid precursors diminished rapidly. This is to be expected, because uric acid is synthesized rapidly in the bird and then excreted; the isotopic concentration of the uric acid thus would reflect directly the isotopic concentration of its precursors. Contrarily, the N^{15} concentration of the glucosamine isolated after the cessation of feeding $N^{15}H_4Cl$ dropped off more slowly. Thus glucosamine after synthesis is utilized less rapidly in the bird than is uric acid. These data can be interpreted as meaning that the glucosamine used for ovomucoid

TABLE I

Degradation of Uric Acid after Feeding $N^{15}H_4Cl$ to Hen

Nitrogen atom	N^{15} concentration
	atom per cent excess
Uric acid (total)	2.00
N_7	0.68
$N_1 + N_3$	2.03
$N_7 + N_9$	1.87
$N_1 + N_7$	0.85
N_1	1.02
N_3	3.04
N_9	3.06

The N^{15} concentration of the various nitrogen atoms of uric acid was calculated as follows: $N_1 = 2(N_1 + N_7) - N_7$, $N_3 = 2(N_1 + N_3) - N_1$, $N_9 = 2(N_7 + N_9) - N_7$.

synthesis is either in equilibrium with the general body stores of glucosamine or at least with a pool which is used for ovomucoid synthesis. If the glucosamine were synthesized for immediate use in ovomucoid synthesis, its isotopic concentration would be expected to have diminished as rapidly as did that of the uric acid after cessation of the administration of the $N^{15}H_4Cl$.

Since the data indicated that a precursor of uric acid may well be a precursor of the nitrogen of glucosamine, the uric acid collected at the point at which maximal incorporation of N^{15} into glucosamine occurred was degraded. The results of this degradation are shown in Table I. The value of the glucosamine isolated at this point was 1.83 atom per cent excess N^{15}. The low values obtained for atoms 1 and 7 of the uric acid would presumably exclude aspartic acid and glycine as precursors for the nitrogen of the glucosamine. Although the values for atoms 3 and 9 of the uric acid are high compared to that for glucosamine, they are close enough

to the glucosamine value to be consistent with the point of view that all three nitrogen atoms share a common nitrogen precursor. This is especially true, since the exact time at which the ovomucoid was synthesized prior to incorporation into the egg is not known and the value of the uric acid is an average of this period. Thus glucosamine may have been synthesized at a time when the value of the isotopic concentration of ammonia incorporated into atoms 3 and 9 of the uric acid was somewhat lower than in the sample actually degraded. Since the amide nitrogen of glutamine is the actual precursor of atoms 3 and 9 of uric acid, these results would imply that the amide group of glutamine is also involved as the direct precursor of the nitrogen atom of glucosamine. This is consistent with the known fact that glutamine may be readily synthesized in animal tissues from glutamic acid and ammonia (24). Although these data cannot determine whether ammonia or glutamine is the actual donor of the amino group of glucosamine, there is no reason to assume that the mechanism of the reaction is any different from that suggested by enzymatic experiments (1, 7, 8).

Feeding of N^{15}-Glycine-1-C^{14}—Although it has been shown that the carbon skeleton of glucose is used intact for the synthesis of glucosamine in the hen, the similarity of the location of the amino group in the 2 position of amino acids and glucosamine suggested the possibility that glucosamine might also be formed by a condensation of an amino acid and a carbon fragment. Such a reaction can be visualized as a condensation of glycine with a tetrose. To test such a hypothesis N^{15}-glycine-1-C^{14} was fed to a hen, and the glucosamine was isolated as before. Since glycine is incorporated intact into uric acid, the uric acid could again serve as an index of the isotopic concentration of the glycine available intracellularly for synthetic purposes. If glycine *per se* is an actual precursor of glucosamine, the C^{14}:N^{15} ratio should be the same in the glycine isolated from the uric acid as that in the glucosamine.

Eggs were laid 29, 74, 102, 146, 175, and 218 hours after the beginning of the feedings. The data obtained are given in Table II. The ratios of C^{14}:N^{15} in the glycine isolated from three samples of uric acid were 38,900, 38,700, and 33,300 c.p.m. per mmole per atom per cent excess N^{15} compared to the initial ratio of 56,700 present in the glycine administered, while ratios in the glucosamine isolated at comparable times were 1330 and 1090. These data indicate that glycine was not used intact for glucosamine synthesis and that the nitrogen of glycine was used more readily than was its carbon chain.

Turnover Rate—At the cessation of the feeding of the isotopic compounds, the decay rate of glucosamine could be determined by the measurement of the isotopic concentration of glucosamine isolated from successive eggs.

TABLE II

Isotopic Concentration of Glucosamine, Uric Acid, and Glycine (Derived from Uric Acid) after Feeding N^{15}-Glycine-1-C^{14} to Hen

Elapsed time after initial feeding	N^{15} (atom per cent excess)			C^{14} (c.p.m. per mmole)	
	Glucosamine*	Glycine	Uric acid	Glucosamine*	Glycine
hrs.					
11	0.028			0	
12			0.51		
24			1.05		
36			0.79		
48		2.28	1.29		88,800
56	0.382			509 (2880)†	
60		2.09	1.16		80,800
Feeding of isotope was stopped					
72		1.34	0.73		44,600
84	0.478			523 (3000)	
128	0.210			180 (1160)	
157	0.160			110 (570)	
200	0.116			70 (407)	

* The glucosamine isotope concentration is recorded at the approximate time of egg white formation.

† The values in parentheses were obtained with a gas flow counter and used for the determination of the half life of the glucosamine. All other counts were obtained with a thin end window Geiger tube.

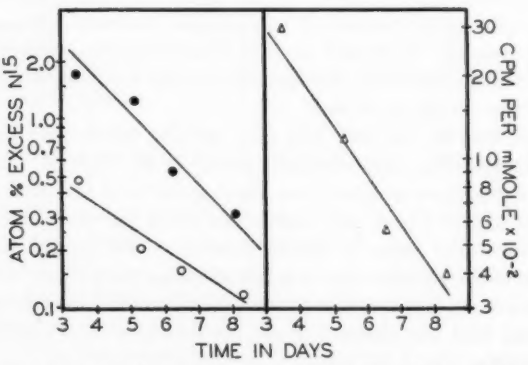

FIG. 2. Decay curves of glucosamine after feeding N^{15}-glycine-1-C^{14} and $N^{15}H_4Cl$. The (○) plot was obtained from the N^{15}-glycine experiment and the (●) plot from feeding $N^{15}H_4Cl$. The (△) plot was obtained from the glycine-1-C^{14} experiment. The time is plotted as that approximated for egg white formation.

This decay rate followed first order kinetics. This was true for both the N^{15} and the C^{14} experiments. The method of least squares was used to obtain the slope of the semilog plot of isotopic concentration *versus* time (Fig. 2). This value was used to calculate the velocity constant, and it, in turn, was used to determine the half life values. In the experiment in which $N^{15}H_4Cl$ was fed, a value of 1.8 days was obtained, while a value of 2.3 days was obtained when N^{15}-glycine was used. The specific activity of the glucosamine-C^{14} determined with a gas flow counter permitted the calculation of the half life of the carbon chain of glucosamine. This value was found to be 1.6 days. The agreement of the values obtained for both nitrogen and carbon of glucosamine indicates that the entire molecule of glucosamine used for ovomucoid synthesis has a half life of about 1.6 to 2.3 days.

The authors would like to express their appreciation to Dr. Sidney Weinhouse and the Houdry Process Corporation, Marcus Hook, Pennsylvania, for the N^{15} analyses, and to Dr. H. Brandenberger for the alkaline peroxide degradation of the uric acid.

SUMMARY

The origin of the nitrogen atom of the glucosamine isolated from the ovomucoid of the egg white has been investigated by the feeding of N^{15} compounds. It has been shown that fed $N^{15}H_4Cl$ was as readily incorporated into the glucosamine as into the uric acid. The correspondence of the concentration of N^{15} in the glucosamine and in positions 3 and 9 of uric acid after administration of N^{15}-labeled ammonium salts suggests that either glutamine or ammonia may be an intermediate in the synthesis of glucosamine in the bird.

N^{15}-Glycine-1-C^{14} was fed to a hen. Although the nitrogen of the fed glycine could be utilized for glucosamine synthesis to some extent, its carbon was not.

The decay curves obtained after cessation of feeding N^{15} and C^{14} followed first order kinetics and permitted the calculation of the half life of the glucosamine. A value of 1.6 to 2.3 days was found.

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INTRAMITOCHONDRIAL NUCLEOTIDES*

I. SOME FACTORS AFFECTING NET INTERCONVERSIONS OF ADENINE NUCLEOTIDES

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(Received for publication, November 26, 1957)

Ample evidence exists that the vital properties of mitochondria are influenced by factors other than the intrinsic properties of their component enzymes. Accordingly, two approaches to the study of mitochondrial function can be differentiated. One may elect to proceed by isolating purified enzymes or simplified enzyme systems, the disengagement of which from the modifying influences of the mitochondrial structure facilitates accurate determinaton of their biochemical and physical properties. The drawback here is that the more purified the material, and consequently the more precise the study possible, the less the certainty about its properties under conditions existing within the living, functioning mitochondrion. Alternatively, study techniques applicable to intact mitochondria can be improved, yielding information containing both enzymic and structural factors which must then be resolved. The work to be presented has been guided primarily by this latter approach. The mitochondrial process of principal concern, oxidative phosphorylation, has been cited particularly often as susceptible to modification by structural alteration of mitochondria (1–5).

It had been observed earlier that, when mitochondria carry out oxidative phosphorylation with added AMP[1] or ADP as the ultimate phosphate acceptor, an abrupt inhibition of both oxidation and phosphorylation sets in 5 minutes after the addition of the adenine nucleotide (7). This inhibition can be overcome by conditions retarding the accumulation of ATP; yet the time interval between the addition of the adenosine nucleotide and the onset of the inhibition cannot be altered by wide variations in the concentrations of AMP, ADP, and ATP, as long as some AMP or ADP remains available to be phosphorylated. This suggested that the key to

* Supported by grants from the American Cancer Society and the National Institutes of Health.

[1] The following abbreviations are used: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; "AD" and "ADX," the two unidentified nucleotides designated as such by Siekevitz and Potter (6); TPN, triphosphopyridine nucleotide; P, inorganic phosphate; CMP, cytidine monophosphate; DNP, 2,4-dinitrophenol.

the mechanism of the adenylate inhibition phenomenon lay in the status of the *intra*mitochondrial nucleotides and their relationship to the extramitochondrial nucleotides. Accordingly, the present investigation was undertaken.

Thus far, mostly negative data have been obtained concerning the adenylate inhibition phenomenon, but many useful techniques and facts have resulted from the study which have revealed hitherto unsuspected degrees of complexity of mitochondrial organization. The present paper will be confined to consideration of some factors governing changes in the mitochondrial adenosine nucleotide levels and attempt to develop general concepts of mitochondrial physiology from these factors.

EXPERIMENTAL

Methods

Rat liver mitochondria were prepared by a modification of the method of Schneider (8) in 0.25 M sucrose containing 0.001 M Versene, pH 7.4. The liver was homogenized in 2 volumes of sucrose-Versene in a Teflon-glass tissue grinder. By calibration of the Servall high speed head according to the gravity-minute procedure of de Duve and Berthet (9), it was possible to work up 50 to 70 gm. of tissue in about 2 hours.

For incubation experiments, mitochondria were added to the reaction mixture, giving a final concentration equivalent to 0.5 gm. of original liver per ml., the final volume being either 12 or 24 ml. One or two 10 ml. aliquots were withdrawn at the appropriate time intervals by means of a calibrated pipette fitted with a rubber bulb, quickly cooled, and gently layered over a sucrose gradient in a 30 ml. plastic centrifuge cup of the International centrifuge high speed head. The sucrose gradient consisted of three unmixed layers of the following composition: bottom layer, 0.02 M KF, 0.001 M Versene, pH 7.4, sucrose to a final specific gravity of 1.10; intermediate layer, 0.001 M Versene, sucrose to a final specific gravity of 1.08; top layer, 0.001 M Versene, sucrose to a final specific gravity of 1.06. The tubes were spun with minimal delay at maximal velocity (about 30,000 $\times$ g) for 4 minutes. When required, a 1 ml. aliquot was carefully withdrawn from the surface layer, following centrifugation, for extramitochondrial nucleotide analysis. The remainder of the medium and sucrose layers were removed by aspiration, and the mitochondrial pellet was briefly rinsed with cold sucrose. Since the sucrose layers remain intact during the centrifugation, the mitochondria are, in effect, thrice washed during their passage through the gradient. The efficiency of this procedure in freeing mitochondria from contaminating medium was established by labeling the medium with glucose-1-$P^{32}O_4$ to which mitochondria are im-

permeable. Less than 0.2 per cent of the total counts added was found associated with the pellet. The nucleotides were then extracted from the mitochondrial pellet with 5 ml. of cold 0.5 M $HClO_4$ with the aid of a spherical glass homogenizer prefitted to the contours of the centrifuge tube. The $HClO_4$ residue was washed with a second 5 ml. portion of $HClO_4$; the combined extracts were neutralized to phenol red with 5 M KOH and allowed to stand at 0° for at least an hour to aid precipitation of the $KClO_4$. Finally the supernatant fractions were decanted onto Dowex 1 columns and chromatographed by a modification of the procedure of Siekevitz and Potter (6).

The columns consisted of glass tubes (1 cm. inside diameter) with sealed in fritted glass disks; they were packed with resin to a height of 3 cm. and eluted with 6 ml. fractions according to the scheme given in Fig. 1. Since the properties of the resin varied considerably from batch to batch, it was necessary to modify the solvent elution schedule anew for each batch of resin. These variations in resin batches are inherent in the resin itself and are not altered by variations in the procedures by which the resin is broken in and purified. Indeed, certain batches of resin meeting the manufacturer's standards proved totally unusable for nucleotide analysis, owing to poor resolving ability or the high background absorption imparted to the column eluents. The nucleotide content of the column eluents was determined by the ultraviolet absorption at 265 mμ rather than at the more conventional 260 mμ in order to minimize the ultraviolet absorption of formic acid. When it was desired to characterize nucleotide bands by spectral ratios, the 280:265 ratio was determined, as it is characteristically different for each of the common nucleotide bases.

Inorganic phosphate was determined by the King method (10) and mitochondrial nitrogen by direct nesslerization.

Materials

Dowex 1-X10 resin, chloride form, 200 to 400 mesh, was either obtained in the purified form from the Bio-Rad Corporation and converted to the formate by washing with 3 M sodium formate or obtained in raw form directly from The Dow Chemical Company and prepared as follows: The crude resin was put through two hydroxyl and chloride cycles with NaOH and HCl, following which the resin was floated on strong sodium formate solution. The sedimenting deep colored heavier material was discarded. The resin was then washed thoroughly with 3 M sodium formate and sized by repeated sedimentation in water so as to remove about 25 per cent of the finer meshed resin, as well as about 5 per cent of the coarser meshed and deeply colored material. 7 ml. eluents required about 17 minutes to drain through columns of the purified resin.

Hexokinase, grade "crude type II," was obtained from the Sigma Chemical Company. The pure nucleotides used in the incubation experiments or in "spotting" chromatograms were from the Pabst Laboratories.

Results

Owing to variations among mitochondrial preparations, it was deemed advisable to present data obtained from a series of experiments conducted on a single preparation rather than to attempt comparisons of experiments done with different preparations on different occasions. Two experimental series embracing the most pertinent sets of conditions were selected for presentation, but the conclusions based on these are consistent with many other experiments.

Complete Chromatogram—The upper chromatogram of Fig. 1 shows the complete nucleotide pattern obtained from mitochondria prior to incubation. The principal components of the various bands are indicated as well as the solvent elution schedule. It is not essential for the inferences which will be drawn from such chromatograms that the bands be pure or homogeneous, since it is improbable that any impurities would undergo band shifts identical to those of the components being studied. Thus by comparison of band shifts rather than the absolute band magnitudes, absorptions due to other components not undergoing changes will largely tend to cancel out.

For comparison the nucleotide pattern of a corresponding aliquot of mitochondria, which had been incubated in a medium of free phosphate acceptor, is given in the lower chromatogram of Fig. 1. The principal effects of such an incubation are the decrease of the AMP and ADP bands and the corresponding increase of the ATP band as compared to the original mitochondria. The diminution of the "AD" and "ADX" bands and the related increase in the TPN band are rendered accountable by establishment of the origin of the main components of these bands as acid breakdown products of dihydrodiphosphopyridine and dihydrotriphosphopyridine nucleotides, respectively (11).

To facilitate comparisons within the experiments, the data obtained by integration of chromatograms analogous to those of Fig. 1 have been converted to bar graph form. Thus, in Fig. 2, Experiments A and D are derived from the lower and upper chromatograms, respectively, of Fig. 1. When it is intended to refer simultaneously to the analogous experiments of both Fig. 2 and Fig. 3, an experimental series is designated by letter; *i.e.*, Series A implies Fig. 1, Experiment A, and Fig. 2, Experiment A, considered collectively.

Substrate-Free Systems—If the substrate is omitted from the complete phosphorylating system (Fig. 2, Experiment B), the added ATP in the

medium is only slightly less effective in phosphorylating the intramitochondrial nucleotides than when substrate is present (*cf.* Fig. 2, Experiment

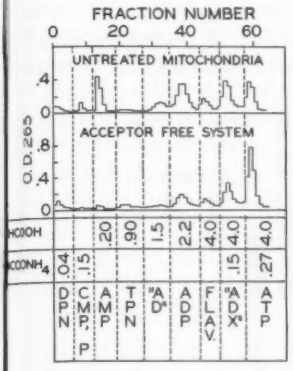

FIG. 1

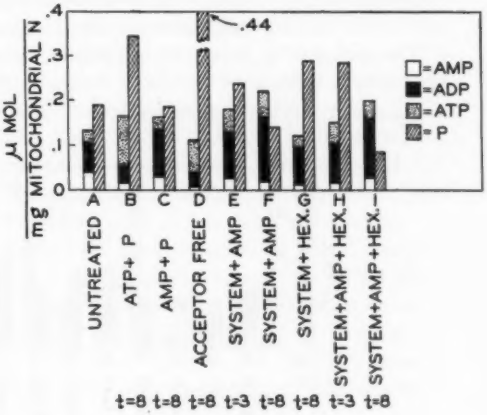

FIG. 2

FIG. 1. Chromatography of mitochondrial nucleotides. The figures describing eluents represent molarity of formic acid or ammonium formate. Ammonium formate prepared by titrating known molarity of formic acid with ammonium hydroxide to pH 5.0. Lower chromatogram obtained by acid extraction of freshly prepared mitochondria; the conditions for incubation of mitochondria used in upper chromatogram given under Fig. 2, Experiment D, and procedure of extraction described under "Methods." The principal components of the chromatographic bands are indicated.

FIG. 2. Experiment A consists of nucleotides extracted from untreated mitochondria (11.8 mg. of N) derived from 5 gm. of original liver tissue. In Experiments B through I, mitochondria (11.8 mg. of N per 10 ml. aliquots) were incubated in a medium containing the following as final concentrations: 0.022 M KCl, 0.0045 M $MgCl_2$, 0.010 M KPO_4, pH 7.4, and 0.20 M sucrose and were shaken at 30° for the indicated time following the last addition. To Experiment B was also added 0.0017 M ATP and to Experiment C 0.0017 M AMP. A stream of moist O_2 was blown across the surface of the reaction mixtures of Experiments D through I, and their medium was supplemented with 0.0017 M ATP and 0.010 M α-ketoglutarate. After a 5 minute preincubation period of the mitochondria, further additions were made as follows: Experiments E-F and H-I, 0.017 M AMP; Experiments G and H-I, 0.03 M glucose plus 0.6 mg. per ml. of hexokinase.

D). Conversely, if ATP is replaced by AMP (Fig. 2, Experiment C), there is a shifting of nucleotides towards the less phosphorylated species, but, even though the added nucleotide is AMP, ADP predominates in the mitochondria. In Fig. 3, Experiment B and Experiment C have been repeated, this time without phosphate in the medium. The results resemble those of Fig. 2. The phosphorylation brought about by ATP,

however, is less complete, while AMP produces a more complete dephosphorylation inasmuch as AMP rather than ADP predominates (*cf.* Fig. 2, Experiment C). There is also some indication that mitochondria retain less of the adenine nucleotides in the absence of phosphate.

The amount of inorganic phosphate found associated with the mitochondria is influenced by many factors. Some loss of phosphate occurs occasionally during centrifugation of unincubated mitochondria through

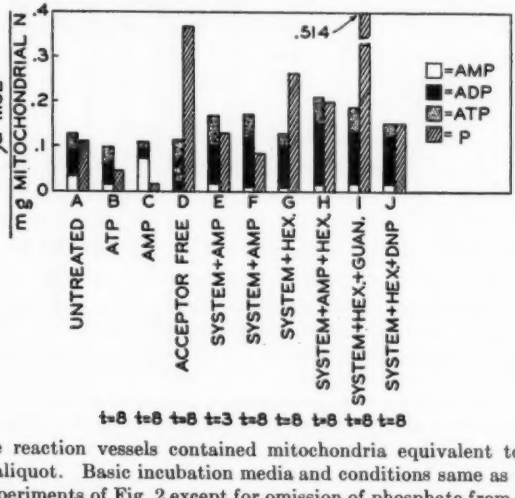

FIG. 3. The reaction vessels contained mitochondria equivalent to 7.8 mg. of N per 10 ml. aliquot. Basic incubation media and conditions same as those of corresponding experiments of Fig. 2 except for omission of phosphate from Experiments B and C. After the preincubation period, further additions were made as follows: Experiment E-F, 0.01 M ATP; Experiments G, H, I, and J, 0.03 M glucose plus 0.6 mg. per ml. of hexokinase; Experiment H, 0.006 M AMP; Experiment J, 5×10^{-5} M DNP. In Experiment I 0.025 M guanidine chloride was present during the preincubation.

the sucrose gradient. The intramitochondrial P is further influenced by the concentration of P in the incubation medium (*cf.* Fig. 2, Experiments B and C, with added phosphate and Fig. 3, Experiments B and C, without added phosphate). Without evaluating the extent to which the apparent differences are due to actual changes in the intramitochondrial P concentration or changes in the ability of mitochondria to retain P during passage through the sucrose gradient, in each case one can observe that mitochondria incubated in the absence of substrate with ATP (Series B) contain considerably more P than those of the analogous AMP-containing experiment (Series C).

Complete Systems with Added Phosphate Acceptors—If AMP is added to

the system free of phosphate acceptor, *i.e.* Series E and F, there is some shift of adenosine nucleotides towards the less phosphorylated forms (*cf.* Series D), but not as extensive as in the absence of substrate (*cf.* Series C). The distribution of nucleotides is not significantly altered if glucose-hexokinase substitutes for (Series G), or is added along with (Series H and Fig. 2, Experiment I), AMP, despite the considerable alterations in extramitochondrial nucleotide distributions introduced in this manner.

Supplementing the medium with AMP (Series E, F, and H) increases the total quantity of intramitochondrial nucleotides (*cf.* Series D and G). This relationship between the magnitude of the intramitochondrial and extramitochondrial adenine nucleotide pools is not simple however. In Fig. 2, although the adenine nucleotide concentration in the medium is over 5-fold greater in Experiment F than in Experiment D or Experiment G, the intramitochondrial level is less than doubled. This net infusion of adenine nucleotides into mitochondria is a relatively slow process, as can be seen by comparing Series E with Series F and, in Fig. 2, Experiment H with Experiment I. The increase of intramitochondrial nucleotides is due primarily to an increase of ADP.

Preliminary experiments have indicated that added AMP cannot increase the intramitochondrial nucleotides in the absence of substrate or in the presence of DNP. This suggests that actively phosphorylating mitochondria, which would therefore be capable of converting AMP to ADP, are a prerequisite condition for the net accumulation of adenine nucleotides within the mitochondria. Studies with isotopically labeled nucleotides, which will be reported on in a subsequent paper, preclude relating this slow infusion process to mitochondrial permeability, since, even at 0°, the intra- and intermitochondrial adenine nucleotides are in an extremely rapidly equilibrating dynamic state.

Phosphorylation-Inhibited Systems—The adenylate inhibition phenomenon is illustrated in Fig. 4 with data obtained from the same reaction mixtures employed in Fig. 2 by periodic removal of aliquots for phosphate analysis. It is apparent from the AMP curve that the mitochondria analyzed in Fig. 2, Experiment F, had, indeed, entered this inhibitory phase, but that the mitochondria of Experiment E had not yet done so. As expected from previous experiments, the inhibition did not show up in the curves marked hexokinase-glucose or AMP + hexokinase-glucose corresponding to Fig. 2, Experiment G or H, respectively. Data obtained in a similar fashion established that analogously, in Fig. 3, Experiment F, but not Experiments E, G, and H, represented mitochondria from this inhibitory phase. No obvious reflection of the adenylate phosphorylation inhibition could be seen by comparison of the experimental Series F with actively phosphorylating mitochondria.

The adenylate inhibition can also be prevented or reversed by 3×10^{-5} M DNP, and it is attenuated by surface active agents, such as deoxycholic acid, known to impair phosphorylation. The possibility of inhibition due to the gradual accumulation of soluble inhibitory products of metabolism is rendered unlikely by the fact that the duration of the induction period is unmodified by variations in the mitochondrial concentration above a critical level of about 0.5 mg. of mitochondrial nitrogen per ml., below which the phenomenon fails to appear.

Hollunger has reported that high concentrations of guanidine exert an inhibitory effect on mitochondria similar to the adenylate effect described above, in that, after a short time delay, both respiration and phosphorylation are inhibited and that respiration can be restored by DNP, although

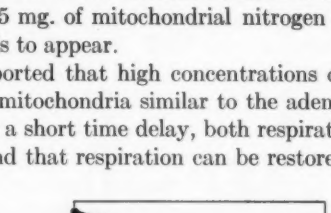

FIG. 4. Phosphorylation rates with different phosphate acceptors. Aliquots were taken at 1 minute intervals from the appropriate reaction vessels also used in Fig. 2. Thus the AMP curve was obtained from Experiment E-F, the hexokinase-glucose curve from Experiment G, and the AMP + hexokinase-glucose curve from Experiment H-I.

in this case not by hexokinase-glucose (12). Since the inhibitions induced by guanidine and adenylate have many points in common, it was of interest to compare the mitochondria under the influence of each agent. As in the case of AMP (Series F) guanidine inhibition produced no significant alteration of the nucleotide pattern (Fig. 3, Experiment I). Guanidine did, however, produce a marked increase in the intramitochondrial inorganic phosphate content. The procedures described have yielded no further clues about the mechanisms of these inhibitions, but the accumulation of inorganic phosphate induced by guanidine, but not by AMP, suggests that the two phenomena are basically different.

A third method of inhibiting phosphorylation was the addition of DNP. The resulting situation differs from the previously described systems, however, since this agent greatly stimulates mitochondrial ATPase, which, in itself, might be expected to alter the intramitochondrial nucleotide distribution. Furthermore, DNP fails to inhibit respiration.

It was surprising to find that DNP (Fig. 3, Experiment J) causes the

conversion of intramitochondrial ATP to ADP and not to AMP as previously reported (6). This experiment has been repeated some half-dozen times with the same result. The extensive effects of DNP on the more dynamic interrelationships between extra- and intermitochondrial nucleotides will be dealt with in another paper.

A second effect of DNP was to bring about a loss of intramitochondrial phosphate. In Series E through I there has been considerable depletion of inorganic phosphate from the medium owing to esterification during oxidative phosphorylation (*cf.* Fig. 4) and, as has been pointed out, this in itself leads to some depletion of intramitochondrial phosphate. This factor is operating most extensively in Fig. 2, Experiment I, and Fig. 3, Experiment F, and does much to account for the low inorganic phosphate levels observed in these instances. Thus, in Fig. 3 the only experiments containing comparable amounts of phosphate in the medium are Experiment D and Experiment J, and here the phosphate-depleting effect of DNP is apparent. In other experiments, particularly when the concentration of DNP was raised, this effect was even more pronounced. The depletion of intramitochondrial P by DNP has also been reported by Werkheiser and Bartley (13).

DISCUSSION

The technical problems involved in effecting an instantaneous and quantitative separation of actively metabolizing mitochondria from an incubation medium are difficult to overcome completely. In the method here employed artifacts may arise from the effects of the hypertonic sucrose gradient on mitochondria, loss of material by diffusion or gain by adsorption, and interconversion of mitochondrial nucleotides following separation but before fixation. These factors are all difficult to assess. Nevertheless, technical improvements and extension of experimental scope have been made as compared with the initial work of Siekevitz and Potter (6).

Since the inception of the present work two additional methods for rapidly separating mitochondria from an incubation medium have appeared which offer certain advantages of rapidity of fixation (13) and simplicity (14), but these in turn have drawbacks in other respects. The most valid basis for appraisal of the techniques described in this paper is probably the consistent and reproducible picture of mitochondrial properties which can be drawn from the data so obtained.

The following conclusions about mitochondrial physiology may be abstracted from the experiments which have been detailed.

The total concentration of intramitochondrial adenine nucleotides is determined primarily by the inherent properties of the particles and is not easily influenced by the extramitochondrial adenine nucleotide concentration.

Under the conditions of active phosphorylation and high extramitochondrial adenine nucleotide concentrations, there is a slow build up of the intramitochondrial adenine nucleotide concentrations, chiefly as the species ADP. The infusion of adenine nucleotides into the mitochondria is greatly inhibited by the addition of DNP, failure to provide a sufficient concentration of extramitochondrial nucleotide, or omission of a phosphate-acceptor system.

The distribution of adenine nucleotides can be influenced considerably by the extramitochondrial adenine nucleotide distribution. The former then reflects the degree of phosphorylation of the latter.

When actively phosphorylating in the presence of a phosphate-acceptor system, the intramitochondrial adenine nucleotide distribution remains essentially unaltered over wide variations of extramitochondrial adenine nucleotide distributions. Such variations were most conveniently provided by permitting the system to phosphorylate over a time interval sufficient to shift the extramitochondrial nucleotides towards the more phosphorylated forms or by comparison of a system without hexokinase-glucose with one otherwise identical but with hexokinase-glucose to maintain the adenine nucleotides in a less phosphorylated state.

The intramitochondrial adenine nucleotides are not freely subject to an adenylate kinase type of equilibrium. This is supported by the failure of a dephosphorylation of intramitochondrial ATP to ADP, either by the action of AMP in the presence of phosphate and absence of substrate (Fig. 2, Experiment C) or by the failure of DNP (Fig. 3, Experiment J) to cause any considerable accumulation of AMP. The discrepancies between this conclusion and an opposing one reached by Siekevitz and Potter (6) are possibly accounted for by the different techniques employed and differences in the original state of the mitochondria.

The intramitochondrial phosphate concentration, in distinction from that of the adenine nucleotides, varies with the extramitochondrial concentration (*cf.* Fig. 2, Experiments B and C, with Fig. 3, Experiments B and C), but other factors are superimposed on this.

In the absence of substrate, both in the presence or absence of extramitochondrial phosphate additions, ATP maintains higher intramitochondrial phosphate concentrations than does AMP. Incubation of mitochondria in the presence of the complete, acceptor-free system also increases the intramitochondrial phosphate (*cf.* Fig. 2, Experiment A, with Fig. 2, Experiment D), which is apparently another reflection of the ATP effect on intramitochondrial P.

The intramitochondrial phosphate concentration is also affected by agents which influence oxidative phosphorylation. Thus DNP causes some loss of intramitochondrial phosphate, while guanidine enhances the accumulation of P by the mitochondria.

Despite the limited effects of the extramitochondrial adenine nucleotide concentration on the intramitochondrial concentration, other experiments with isotopically labeled nucleotides have demonstrated that there is an extremely dynamic interchange of nucleotides between the mitochondria and the medium. These findings would be reconciled by the hypothesis that there exists within the mitochondria a fixed quantity of some integral component of the mitochondria which has an affinity for adenine nucleotides strong enough to prevent their loss by washing. Nucleotide in excess of that needed to saturate this component is either easily washed out of the mitochondria or else fails to accumulate within the mitochondria for other reasons not yet understood. The affinity between adenine nucleotides and this hypothetical component is great enough, however, to interfere with the free interconversion of the different phosphorylated species of the adenine nucleotides both within and without the mitochondria. Thus the bound molecules of adenine nucleotide would be susceptible to easy displacement by free nucleotide molecules entering the mitochondria from the medium. The binding component may, however, exert enough influence on the intramitochondrial nucleotides to account for those cases cited in which the distribution of intramitochondrial adenine species is markedly different from the extramitochondrial distribution.

SUMMARY

1. Techniques have been described for the rapid and complete separation of mitochondria from an incubating medium. Improved methods have been developed for the extraction and resolution of the mitochondrial nucleotides.

2. The effects of several incubation conditions, such as variation of phosphate-acceptor systems and addition of inhibitors of phosphorylation, on the intramitochondrial adenine nucleotide distribution have been described.

3. The intramitochondrial nucleotide distribution and concentration were found to be no simple function of the extramitochondrial nucleotide distribution and concentrations, but depended for the most part on other factors within the mitochondria.

4. Some attempt has been made to chart those physiological properties of mitochondria responsible for governing the interconversion of adenine nucleotides both across the mitochondrial membrane and within the mitochondria.

The author wishes to thank Dr. H. A. Lardy for many valuable suggestions and criticisms during the course of this work and the preparation of this manuscript. The technical assistance of Miss Katherine Mueller and Mr. Albert Kuschel is also gratefully acknowledged.

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THE QUANTITATIVE HISTOCHEMISTRY OF THE BRAIN*

V. ENZYMES OF GLUCOSE METABOLISM

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(Received for publication, January 22, 1958)

The importance of glucose for nervous function has long been recognized. This paper is a report of the histochemical distribution in certain parts of the brain of seven enzymes which are known to act upon hexose or hexose phosphates. The distribution of these seven enzymes might be expected to signify for each histological element the relative capacity and possible importance of four main channels of glucose utilization: (*a*) primary glucose esterification (hexokinase[1]), (*b*) glycogen metabolism (phosphorylase and phosphoglucomutase), (*c*) glucose metabolism via the Embden-Meyerhof system (phosphoglucoisomerase or isomerase, phosphofructokinase, and aldolase), and (*d*) oxidation via the "shunt" (glucose-6-phosphate dehydrogenase).

The distribution of the seven enzymes was measured among ten histologically segregated zones of the central nervous system of the rabbit: two molecular layers, a layer of dendrites, two layers of packed cell bodies, and five myelinated fiber tracts.

It was not practical to obtain large samples of these zones. Therefore it was necessary to elaborate microchemical methods in the case of the five enzymes for which sufficiently sensitive methods were not already available. The resulting procedures require only 0.1 to 5 γ of dry brain and have been validated by a variety of tests, including the recovery of the activities of purified enzyme preparations when added to whole homogenates and the assessment of the stability of these enzymes in rabbit brain to freezing, drying, and storage.

* Supported in part by a grant-in-aid from the American Cancer Society.

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[1] The abbreviations used are Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides; ATP, adenosine triphosphate; HK, hexokinase; PGM, phosphoglucomutase; PFK, phosphofructokinase; G6DH, glucose-6-phosphate dehydrogenase; AMP, 2-amino-2-methylpropanediol; LDH, lactic dehydrogenase; and PHRL, glycogen phosphorylase.

At least some of the procedures developed might find application for more general purposes, since they are rather convenient and quite reproducible. The isomerase method is based on the measurement of the F6P formed. Changes made in the fructose method of Roe *et al.* (1) seem to constitute a substantial improvement. For the measurement of PGM, conditions were established for hydrolysis of G1P and measurement of the

TABLE I

Incubation Conditions for Six Enzymes Measured

	pH	Buffer		Substrate		Other additions
			M		mM	
Hexokinase	8.0	Hydrazine	0.04	Glucose	7	Mg 5 mM; KH_2PO_4 0.015 M; purified isomerase + PFK + aldolase
				ATP*	3	
Phosphoglucoisomerase	8.0	Tris	0.1	G6P	90	
Phosphofructokinase	8.0	K_2HPO_4	0.04	F6P	7	Hydrazine 0.03 M; Mg 6 mM; $(NH_4)_2HPO_4$ 1.3 mM; crystalline aldolase 0.01%; crystalline bovine albumin 0.02%
				ATP	15	
Phosphorylase	6.7	Substrate		G1P	30	Glycogen 0.5%; 5′-adenylic acid 1 mM; cysteine 15 mM; NaF 50 mM
Phosphoglucomutase	7.6	Tris	0.02	G1P	1	Mg 1.5 mM; BAL 2 mM; glucose 1,6-diphosphate, 2×10^{-6} M
G6P dehydrogenase	8.9	AMP†	0.1	G6P	5	Mg 10 mM; Versene 1 mM; TPN^+ 2 mM

* It would be preferable to increase ATP to 10 mM.

† 2-Amino-2-methylpropanediol.

P_i in one step. For the measurement of phosphorylase, improved conditions were found for measuring P_i in the presence of G1P.

Materials and Methods

The analyses were all performed on histologically characterized fragments dissected from frozen-dried sections of rabbit brain or spinal cord. The preparation and weighing of such fragments before analysis have been described (2). The general techniques and tools for measuring enzymatic activities on the required microscale, as well as a description of the aldolase method, have been given in detail (3, 4). The method for G6DH is taken

from an unpublished procedure of Dr. A. W. Albers. After incubation under the conditions given in Table I, protein is precipitated by adding 0.33 M KCl in 67 per cent ethylene glycol monomethyl ether, and the TPNH formed is measured by its absorption of light at 340 mμ. The lipide was measured by loss of weight upon extraction with alcohol and hexane (2).

All of the analyses to be presented here were made with tissues from a single rabbit. However, the results have been confirmed by less complete data from other rabbits, and whole brain homogenates have been analyzed upon innumerable occasions for all of these enzymes. The disparity among the animals was found to be remarkably small.

All of the analyses were performed in the same manner. The dissected and weighed samples (1 to 2 γ) were placed in tubes in racks and stored at −20° until they could be analyzed. At this time the racks were placed in an ice bath, and a measured volume of complete buffer-substrate reagent was added to each tube. The entire rack of tubes was then incubated at 38°, and the action was arrested in an ice bath before subsequent steps.

All of the methods have been tested extensively with whole brain homogenates for effects of variations in pH, substrate, or coenzyme concentration, time of incubation, and activity with different dilutions of homogenate. Unless otherwise noted, the incubation conditions were such as to give maximal activity and proportionality between colorimeter readings and either the amount of enzyme or the time of incubation up to at least 1 hour. With reasonable care, the methods are all reproducible to the limits of the spectrophotometer and, in order to conserve space, this fact will not be documented. A more practical test is provided by the over-all reproducibility of the histochemical values to be presented.

Phosphofructokinase

Principle—The reagent contained added crystalline aldolase which converted hexose diphosphate as fast as it was formed to triose phosphates. These were trapped by hydrazine and measured colorimetrically with dinitrophenylhydrazine.

Procedure—Dry samples weighing 1 to 2 γ were incubated with 18 μl. of complete reagent for 30 minutes at 38°. This substrate reagent was made freshly before use (Table I). The aldolase, prepared according to Taylor *et al.* (5), was recrystallized twice, and the wet crystals were stored at −20°. A 1 per cent solution was prepared as needed. The reaction was permanently arrested with 3 μl. of 30 per cent trichloroacetic acid. The samples were centrifuged, but this is probably unnecessary because of the small amount of protein involved. However, acidification cannot be omitted. Aliquots of 10 μl. were analyzed for triose phosphates by a

published procedure (4) adapted from the aldolase method of Sibley and Lehninger. The final volume for reading was 345 μl. For calculation, it was assumed that the extinction coefficient corresponding to 1 mole of F6P converted to triose phosphate would be 47,800. This is based on the value obtained with G6P in the similar system used for the assay of hexokinase (see below). Neither F6P nor hexose diphosphate was available in sufficient purity to use as a standard. A sample calculation is as follows. A particle of tissue weighing 1.5 γ was incubated for 30 minutes as detailed above. The observed net optical density was 0.250. Therefore the activity was calculated to be (21 λ/1.5 γ) × (345 λ/10 λ) × (0.250/47,800) × (60/30) × 1000 = 5.05 moles per kilo per hour.

Comment—Rabbit brain PFK is exceedingly unstable in water homogenates, but may be protected if fresh brain is homogenized (1:10) in 0.2 M phosphate buffer at pH 7.8.

Frozen-dried brain sections, however, appear to be stable almost indefinitely if they are kept cold. Samples from the stratum radiatum of Ammon's horn after 4 years storage at −20° had 85 per cent of the activity of samples stored for 1 month (from another rabbit).

Rabbit brain PFK was purified about 20-fold by the procedure of Muntz (6). When this was mixed with a crude homogenate, the activities were additive. (Observed net optical densities were 0.627, 0.469, and 1.085 for samples containing, respectively, purified enzyme, whole homogenate, and both. The calculated sum was 1.096.)

Hexokinase Assay

Principle—The G6P formed was converted to triose phosphate by added PFK, phosphoglucoisomerase, and aldolase. Hydrazine was used to trap the triose phosphates and to prevent the action of triosephosphate isomerase. The triose phosphates were measured colorimetrically with dinitrophenylhydrazine.

Procedure—The substrate reagent was prepared freshly before use (Table I). However, all components other than ATP and PFK may be combined and stored at −20°. The auxiliary enzymes were prepared as a single mixture (PFK) from skeletal muscle by the procedure of Taylor (7) as modified by Crane and Sols (8). The amount used was sufficient to give not less than 90 per cent conversion of G6P to triose phosphates in 5 minutes.

Dry samples weighing 1 to 2 γ were incubated with 5 μl. of complete reagent for 30 minutes at 38°. The reaction was permanently arrested with 1 μl. of 30 per cent trichloroacetic acid. After centrifugation, 5 μl. were analyzed for triose phosphates, as in the case of PFK, except that all volumes were reduced by half. Standards consisted of 5 μl. samples of 1

mM G6P made up at the last minute in the complete substrate reagent, and these were carried through the entire procedure.

Comment—The removal of G6P as fast as it is formed has the advantage of preventing the marked product inhibition which would otherwise occur (8, 9). Consequently, the color produced was proportional to enzyme concentration to within 5 per cent over a 4-fold range. Also, observed rates were nearly linear with time (10 per cent less at 90 than at 20 minutes).

The color produced per mole of glucose phosphorylated is about 6 times that given by 1 mole of glucose in the usual colorimetric reduction methods. Furthermore, the method is direct and can be employed on a scale which would be troublesome for glucose measurement.

On a semimicroscale, parallel measurements of glucose disappearance and triose phosphate formation gave identical results. Hydrazine was omitted in the first instance because this would have interfered with the glucose measurement.

A sample of brain hexokinase purified 50-fold was generously provided by Dr. Robert K. Crane and Dr. Alberto Sols. By the given procedure, the activities of this sample and of whole brain homogenate were additive. (Optical densities separately were 0.306 and 0.383, respectively; the optical density of the mixture was 0.685 observed, and 0.689 calculated.) Therefore, there were no obvious complications arising from the use of crude brain samples.

Rabbit brain hexokinase is unstable in aqueous homogenates, particularly when highly diluted, and is not preserved by freezing. However, the stability is much better in phosphate buffers at pH 7.5. Even so, attempts to dry small aliquots of frozen homogenates have resulted in loss of over 50 per cent of the activity. This loss may be, in part, the result of failure to disperse aggregated particles for the analysis (Crane and Sols (8)). In spite of the low values with frozen-dried homogenates, it is believed that HK is completely preserved in frozen-dried tissue sections. The values obtained for dried sections from various parts of the brain fall within the range expected from the average for whole brain, and frozen-dried sections stored for 4 years (at $-20°$) have been found to be at least 90 per cent as active as fresh frozen-dried sections.

Phosphoglucoisomerase

Procedure—Dry samples weighing 1 to 2 γ were incubated for 30 minutes at 38° in 10 μl. of reagent (Table I). Aliquots of 7 μl. were transferred into 1 ml. of color reagent in a 3 ml. test tube and, because of the viscosity of the reagent, were vibrated or tapped vigorously in order to mix them. Color was developed by heating the mixture 20 minutes in a water bath at

60°, and readings were made at 500 mμ. Standards, consisting of 10 μl. of reagent containing 5 mM fructose, and blanks were included in all the sets of samples. F6P appeared to give about 80 per cent as much color as fructose, and this value was used in the calculations.

The color reagent consisted of a fresh mixture of 40 volumes of 20 N H_2SO_4 with 1 volume of glacial acetic acid containing 0.4 per cent resorcinol and 1 per cent thiourea.

The color of samples is almost insensitive to light, and readings may be made at any time up to 24 hours. However, blanks and standards must

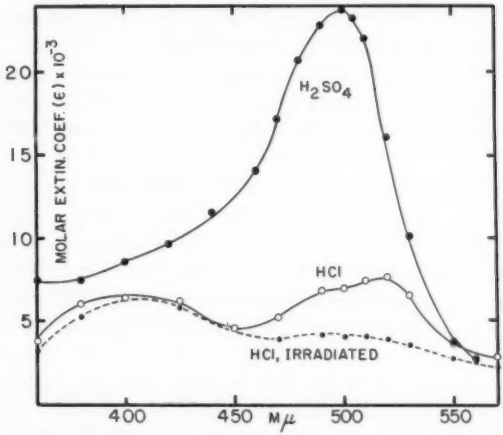

FIG. 1. Absorption spectra of fructose heated with resorcinol in H_2SO_4 and HCl. The conditions of reaction in H_2SO_4 were those proposed in this paper. The conditions of reaction in HCl were those prescribed by Roe *et al.* (1). Irradiation consisted of 60 minute exposure 12 inches from a 100 watt tungsten lamp.

be run at the same time because the color may change slightly, and the blank increases progressively as a result of the G6P.

Comment. Fructose Measurement—The method of Roe *et al.* (1) presented difficulties on a microscale. The HCl concentration is quite critical, and during the heating step (80°) HCl volatilized somewhat from microsamples and changed the composition and the final color in an erratic manner. The sensitivity to light of the color produced was an added source of error. This second difficulty could be largely avoided by reading at 400 instead of at 520 mμ, because the peak at the shorter wave length changes much less upon irradiation (Fig. 1).

The problem of volatility, however, was more troublesome, and H_2SO_4 was therefore substituted for HCl with considerable advantage. The

color produced was two or three times as great as in HCl, was stable to light, and could be developed in 20 minutes at 60° in 20 N H_2SO_4 or in an hour at 38° in 25 N H_2SO_4. The only disadvantage was the viscosity of the sulfuric acid, which was moderate at 20 N or below, but which increased much more rapidly above 20 N. Curiously, a 0.1 volume of glacial acetic acid used as a medium for adding thiourea and resorcinol increased the viscosity of 20 N sulfuric acid by nearly 50 per cent. Therefore, the proportion of acetic acid was reduced from 10 per cent, as used by Roe *et al.*,

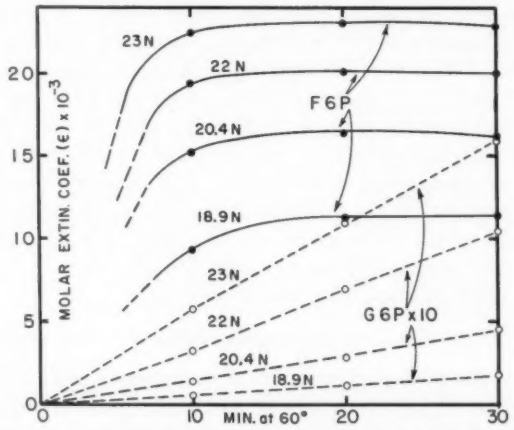

FIG. 2. Extinction coefficients at 500 mμ with F6P and G6P heated for various times with resorcinol in sulfuric acid of indicated concentrations. Note that the scale for G6P is expanded 10-fold.

to 2.5 per cent, with a compensatory increase in the concentration of thiourea and resorcinol in the acetic acid.

A record of the effects of changes in time of heating and sulfuric acid concentration on the color produced with F6P and G6P may be useful (Fig. 2). It is interesting that the color produced in H_2SO_4 differs from that obtained in HCl (Fig. 1). The HCl product is somewhat fluorescent and becomes very fluorescent on irradiation, whereas the H_2SO_4 product is only moderately fluorescent and is not enhanced by irradiation.

Isomerase Properties—Rabbit brain isomerase has a pH optimum at 8, with a decrease to 80 and 88 per cent of maximal activity at pH 7 and 8.5, respectively. The buffer used (Tris) is probably not inhibitory since about the same activity was obtained with half or twice the concentration. The K_m is probably small since the velocity was as fast with 2.5 mM substrate as with 90 mM, provided that the same small fraction of substrate

was isomerized. The velocity decreases as the fraction of the substrate is converted about as much as predicted for a reversible reaction. Consequently, for accurate analytical results, the fraction of substrate converted must be kept small, or a suitable correction curve must be used.[2] Because of the small K_m, the substrate concentration may be adjusted as necessary to keep the fraction of substrate converted within suitable limits.

Brain isomerase withstands freezing and drying without loss. There is a loss of about 50 per cent after a week at 25° in the dry state. At −20°, frozen-dried sections seem to be stable indefinitely (for years).

Isomerase was purified 25-fold from rabbit brain. An extract of brain made with 2 volumes of 0.03 N KOH was combined with a water washing of the residue. This was fractionated with ammonium sulfate at pH 7.5 between 1.6 and 2.4 M, and refractionated between 2.0 and 2.2 M. The product had an activity of about 8000 moles per 100 kilos of protein per minute. A recovery experiment was performed in which this preparation was added to whole brain homogenate. The recovery averaged 120 per cent. The extra activity is attributed to protection of the purified enzyme by the protein of the crude brain. It has since been found that at very high dilutions it is necessary to add inert protein, such as bovine plasma albumin, in order to avoid some loss of activity.

Phosphoglucomutase

Principle—The disappearance of G1P is measured after incubation in a single analytical step in which the Cori ester is hydrolyzed and the liberated phosphate produces maximal color with molybdate and ascorbic acid.

Procedure—Samples of 1 to 2 γ of dry weight were incubated for 20 minutes at 38° in 7 μl. of the substrate solution indicated (Table I). This substrate solution may be stored in a frozen state without the BAL or glucose diphosphate, which can then be added separately as needed. The BAL is conveniently prepared by shaking a 10 per cent solution in peanut oil with 10 volumes of water, which extracts almost 90 per cent and yields a 65 mM solution that is stable in a frozen state for at least a week. The reaction was permanently arrested with 7 μl. of 10 per cent trichloroacetic acid. After centrifugation to remove protein, a 10 μl. aliquot was transferred to a tube of 3 mm. inner diameter (50 or 60 mm. long), and 100 μl. of phosphate reagent were added. The tubes were heated for 45 minutes in a water bath at 50° (well capped with Parafilm to prevent evaporation), and the absorption was measured at 820 mμ. The phosphate reagent is prepared by mixing, just before use, 5 volumes of 10 per cent ascorbic acid

[2] Recently Alvardo and Sols have found that the F6P may be readily trapped with borate without inhibition of isomerase (10). In consequence, the reaction can be made linear until nearly all of the G6P is gone.

with 10 volumes of 1.7 per cent ammonium molybdate in 3 N H_2SO_4. The ascorbic acid solution is stored in a frozen state.

G1P is completely hydrolyzed by the procedure given, and the phosphomolybdate is reduced with a molar extinction coefficient of about 27,000. G6P is about 0.5 per cent hydrolyzed. The reaction can be standardized by the substitution of 7 μl. of 1 mM P_i for the substrate reagent.

Since a decrease in sample reading is the measure of enzyme action and since the reaction departs from linearity if too much substrate is consumed, an effort is made to convert not less than 20 or more than 50 per cent of the G1P to G6P. The size of the sample, the volume of the reagent, and the incubation time may be varied for this purpose. The substrate concentration may also be increased if necessary.

Comment—The only definite change in the assay system as compared to those of previous investigators was the substitution of BAL for cysteine or other metal-binding agents (11). With 0, 0.6, 1.2, 2.2, and 3.4 mM BAL, relative activities were 17, 87, 100, 100, and 100 per cent, respectively. With 12 mM cysteine the activity was 10 per cent less, possibly as a result of the increase in total salt concentration.

With 0, 1.5, and 15 mM Mg, activity was 18, 92, and 100 per cent of that with 4 mM Mg. The activity without added Mg is ascribable to Mg in the brain homogenate, and the data are consistent with a K_m for Mg of about 4×10^{-5} M.

Without added glucose diphosphate, activity was very low and varied with tissue dilution. At whole rabbit brain dilutions of 1:10,000 and 1:1000, activity was 10 and 40 per cent, respectively, of the activity with 2×10^{-6} M glucose diphosphate. With added coenzyme, activity was closely proportional to both time and concentration of brain until 50 per cent of the substrate was converted.

A pH optimum near 7.5 was confirmed for brain PGM. Activities were, respectively, 82 and 90 per cent of the maximum at pH values of 7.2 and 8.0. Because of the sensitivity to pH, dilute buffer was used in the reagent, in spite of the known inhibitory effect of salts.

The activity of brain enzyme at 1° and 25° was found to be 2.1 and 37.1 per cent of that at 38°.

In order to test for possible inhibitory or enhancing factors in whole brain homogenates, PGM was purified 25-fold from rabbit brain and mixed with a crude homogenate. The respective activities were additive to within 2 per cent when assayed in combination. The purification was accomplished with ammonium sulfate fractionation at pH 7.5 of an alkaline brain extract. The fraction, which precipitated between 40 and 60 per cent saturation, was again precipitated between 55 and 60 per cent saturation.

The activity of PGM is stable in frozen-dried sections stored at −20° for at least a year.

Color Development—G1P was hydrolyzed and the color developed from the liberated P_i in one step. This was accomplished at a moderate temperature by taking advantage of the catalytic activity of molybdate on the hydrolysis (12) and by increasing the sulfuric acid concentration to 2 N. As the acidity increases, phosphomolybdate becomes increasingly difficult to reduce. This is offset by the following changes, in comparison to a previously published method for P_i of similar sensitivity which was performed with 0.9 N H_2SO_4 (3). Ammonium molybdate is increased from 0.25 to 1 per cent, ascorbic acid from 1 to 3 per cent, and the temperature is raised from 38–50°. The extinction coefficient is about 6 times that obtained by the method of Fiske and Subbarow. The procedure is clearly useful only in special situations since many phosphate esters would be partially hydrolyzed under the analytical conditions. Under the prescribed conditions, P_i gives 80 per cent full color in 10 minutes, and G1P 80 per cent full color in 25 minutes. If desired, the time for color development may be decreased from 45 to 20 minutes by heating at 57° instead of at 50°.

Glycogen Phosphorylase—The reagent was a fresh mixture of equal volumes of a solution (*a*) containing, at double strength, all of the ingredients indicated (Table I) except cysteine, and a fresh, neutralized (pH 6.7), 0.03 M cysteine solution (*b*) prepared from 0.3 M cysteine hydrochloride. Solution *a* was stored at −20°.

Each tissue sample (1 to 2 γ) was incubated with 10 μl. of complete reagent for 30 minutes at 38°. The reaction was permanently arrested with 1 μl. of 55 per cent trichloroacetic acid with prompt centrifugation in the cold to minimize the acid hydrolysis of G1P. The tubes were kept in ice water as much as possible. Aliquots of 10 μl. of supernatant fluid were promptly added to 50 μl. of phosphate reagent. This consisted of a fresh mixture of 1 ml. of 2.5 per cent ammonium molybdate, 7 ml. of H_2O, 0.02 ml. of 1 per cent $CuSO_4 \cdot 5H_2O$, and 1 ml. of a fresh 6 per cent solution of a powder composed of 95 per cent $NaHSO_3$, 4.5 per cent Na_2SO_3, and 0.5 per cent 1-amino-2-naphthol-4-sulfonic acid (13).

The samples were read at 700 mμ after 10 minutes. Standards consisted of 10 μl. of reagent which was 0.5 mM in KH_2PO_4. Standards and blanks were treated as nearly as possible in the same way as the samples.

Comment. Measurement of Inorganic Phosphate Released—The P_i is measured with a reagent similar to that of Fiske and Subbarow (13), except that sulfuric acid is omitted, and the only acid is the trichloroacetic acid used to precipitate protein (final concentration about 0.05 N). Since the final pH is about 2.5 (instead of 0.6), there is practically no hydrolysis

of G1P, and the color from P_i does not increase with time (Furchgott and deGubareff (14)). Copper is added to accelerate color development, as suggested in 1949 by Dr. C. S. Hanes (personal communication).

If more color is desired, the molar extinction coefficient, ϵ_m, can be increased from about 4000 to 24,000 by using a reagent which contains (at final dilution) 0.2 N H_2SO_4, 0.1 per cent ammonium molybdate, and 3 per cent ascorbic acid. Reading is made after 60 minutes at room temperature (25–28°) or after 20 minutes at 38°. The reagent has some disadvantages. Approximately 1.5 per cent of the G1P is hydrolyzed in this length of time, and the blank increases a little with time, even without G1P present.

Rabbit Brain Phosphorylase Properties and Validation of Procedure—With dilution of rabbit brain 200-fold or more, P_i formation in the absence of added glycogen was not over 2 per cent of that with 0.5 per cent glycogen present. This indicated that, even though the assay was made on whole brain, there were no significant sources of P_i other than that of the phosphorylation reaction. Half maximal activity was obtained with 0.013 per cent glycogen.

When G1P concentration was varied, the activities indicated a K_m of 7.2 mM. Similarly, the activities with different levels of adenylic acid were compatible with a K_m of about 0.05 mM. Activity was enhanced 25 per cent by cysteine, 15 mM, but a lower level of BAL (1.5 mM) was not stimulatory. Versene will not replace cysteine with the brain enzyme. Activity was relatively independent of pH between 6 and 7.

Altogether, brain phosphorylase in the whole tissue assay system behaved about as might be expected from the studies of Cori *et al.* (15) with highly purified preparations.

Phosphorylase in brain homogenates withstood freeze-drying; in fact, the activity increased 35 per cent, probably as a result of better disruption of the tissue. There is no evidence of destruction of activity during storage in the dry state at −20°. A brain stored at −20° for a year without drying retained 60 per cent of the activity of a fresh brain.

P_i liberation was closely proportional to the time of incubation and the amount of brain, provided that not more than 10 per cent of the substrate was used.

RESULTS AND DISCUSSION

The values have been calculated on a lipide-free basis since the wide range of lipide might obscure the significance of the results. The lipide-free dry weight has been found to be a nearly constant fraction (10 to 12 per cent) of the wet weight of various parts of brain, in spite of extreme differences in lipide content.

The results will be examined with three purposes in mind: (1) to compare the absolute activity values for the seven related enzymes, (2) to contrast the histological distribution of enzymes concerned with alternative pathways of hexose utilization, and (3) to ascertain to what degree enzymes of the same metabolic pathway vary in parallel fashion as the metabolic pattern changes throughout the nervous system.

The enzymatic activities in whole brain were all measured under the near optimal conditions which are believed to make the enzyme in question strictly rate-limiting and which avoid significant destructive or inhibitory factors. Therefore, the absolute values may merit examination (Table II). Isomerase leads, with the enormous value of 150 moles per kilo of fat-free dry weight per hour, which is 100 times greater than the value of G6DH, the least active. It must be recognized that these are maximal velocities obtained with high substrate levels at optimal pH for each enzyme. Therefore, in interpreting these activities the actual pH and substrate conditions in the cells would have to be evaluated. For example, because of its small Michaelis constant, it may be that G6DH, in spite of its low absolute activity, may compete with some success with isomerase and PFK for G6P. It is clear that most of the seven enzymes operate at far less than maximal velocity since oxygen consumption of brain does not exceed that necessary to burn 0.2 mole of glucose per kilo of fat-free dry weight per hour, and maximal glycolysis is sufficient only to use 0.6 mole of glucose per kilo per hour (16).

There are modest but significant differences among the five non-myelinated layers (Table II). Nearly all of the enzymes tend to be a little lower in content in the cell body layers, whereas the dendrite layer is particularly rich in isomerase and PFK.

More striking are the differences between the gray layers and the myelinated tracts. Without exception, the white tracts are relatively poor in hexokinase and relatively rich in G6DH. The data suggest that white tracts are more capable of using glycogen than glucose, and that the direct oxidative shunt is a relatively important pathway in the use of G6P. Among themselves, the white tracts differ a good deal. There is indication of an inverse relationship between enzymes of glycolysis and G6DH (PFK *versus* G6DH in Table III), and a direct relationship between total lipide and G6DH (Table III).

Optic tract is exceptional in its high isomerase activity. It was previously found to be very rich in lactic dehydrogenase (17). The retinal ganglion cell layer, containing the cells of origin of the optic tract, is also rich in lactic dehydrogenase in the rabbit (17, 18). It is presumably rich in all enzymes of the glycolytic cycle. The optic tract is the richest in PFK of the five tracts, but by a much smaller margin than in the case of

isomerase or lactic dehydrogenase, and it is only second highest in aldolase activity. Possibly this means that the extraordinary levels of certain enzymes of glycolysis in the optic tract are the consequence of diffusion

TABLE II

Distribution of Seven Enzymes of Glucose Metabolism in Ten Regions of Rabbit Brain

All enzyme values are recorded as moles of substrate transformed per kilo of lipide-free dry weight per hour. The lipide is recorded as percentage of lipide-free dry weight. Each enzyme value represents the average of six analyses as a rule. The standard errors are given in bold-faced type. Lipide values are from earlier studies.

	Lipide	HK	Isomerase	PFK	Aldolase	PGM	PHRL	G6DH
Molecular layer, cerebellum	73	6.70	164	21.3	10.6	9.4	12.9	2.38
		0.42	**7**	**1.9**	**0.3**	**0.4**	**0.3**	**0.06**
" " Ammon's horn	63	6.42	116	15.9	8.4	10.3	8.9	1.30
		0.44	**4**	**1.2**	**0.3**	**0.3**	**0.6**	**0.08**
Dendrite layer, Ammon's horn	61	5.46	193	32.5	9.4	9.0	6.8	1.18
		0.21	**7**	**1.6**	**0.2**	**0.3**	**0.3**	**0.04**
Cell body layer, cerebellum	45	5.09	88	11.0	5.2	5.9	7.3	1.15
		0.20	**3**	**1.1**	**0.1**	**0.3**	**0.1**	**0.03**
" " " Ammon's horn	28	4.32	94	11.1	4.7	5.3	5.4	0.86
		0.15	**3**	**0.6**	**0.1**	**0.2**	**0.2**	**0.03**
White tract, Ammon's horn	161	2.43	82	13.0	5.0	6.0	6.4	1.85
		0.18	**3**	**0.4**	**0.1**	**0.3**	**0.3**	**0.05**
" " cerebellum	240	1.93	95	13.4	7.3	7.4	5.0	3.32
		0.17	**3**	**0.8**	**0.3**	**0.6**	**0.2**	**0.08**
" " optic	251	2.17	234	15.6	6.9	6.6	3.9	2.67
		0.14	**11**	**0.8**	**0.1**	**0.2**	**0.2**	**0.15**
" " dorsal columns	317	1.54	66	11.2	2.6	9.2	5.7	5.42
		0.08	**4**	**0.7**	**0.2**	**0.5**	**0.2**	**0.21**
" " dorsal spino-cerebellar	324	1.38	62	8.4	3.0	6.5	4.4	5.25
		0.04	**4**	**0.4**	**0.1**	**0.5**	**0.4**	**0.21**
Average brain	100	6	150	16	6.5	10	7	1.5

from the avascular ganglion cell layer of the retina rather than indicative of an unusually high glycolytic rate in the optic tract itself.

PFK and PHRL bear a nearly constant ratio in many of the regions of the brain examined (Table III). The ratios are less constant between PFK and HK. This might suggest that maximal capacity for a burst of glycolysis is related to ability to use glycogen rather than to capacity for direct utilization of glucose.

The above discussion presupposes that the local concentration of a particular enzyme is indicative of the capacity for metabolism along the pathway which contains that enzyme. If this is true, then all members of that pathway should vary in constant ratio. The ratios of Table III support this thesis, but only in part. Thus isomerase, PFK, aldolase, and lactic dehydrogenase in general vary in a like manner, but in certain cases there are rather large deviations from the mean ratios. These deviations could indicate some tolerance in the concentration of certain

TABLE III

Ratios between Various Enzymes of Glucose Metabolism in Rabbit Brain

Tissue	PFK / HK	PFK / PHRL	PFK / G6DH	Isomerase / PFK	PFK / Aldolase	Isomerase / LDH*	PGM / PHRL	G6DH / (—0.6 Lipide†)
Molecular layer, cerebellum	3.2	1.6	9	8	2.0	5.8	0.7	26
" " Ammon's horn	2.5	1.8	12	7	1.9	4.6	1.2	11
Dendrite layer, Ammon's horn	6.0	4.8	27	6	3.5	6.0	1.3	11
Cell body layer, cerebellum	2.2	1.5	10	8	2.1	4.1	0.8	14
" " " Ammon's horn	2.6	2.1	13	9	2.4	3.4	1.0	13
White tract, Ammon's horn	5.3	2.0	7	6	2.6	4.5	0.9	8
" " cerebellum	6.9	2.7	4	7	1.8	4.1	1.5	12
" " optic	7.2	4.0	6	15	2.3	4.2	1.7	9
" " dorsal columns	7.3	2.0	2.1	6	2.0	4.1	1.6	15
" " " spino-cerebellar	6.1	1.9	1.6	7	1.6		1.5	15
Average brain	2.3	2.3	11	9	2.5		1.4	20

* Calculated from data previously published (17) on lactic dehydrogenase.

† Gm. of lipide per gm. of lipide-free dry weight. This empirical relationship gives the best graphical fit of a linear plot of G6DH *versus* total lipide.

enzymes of a series, or might indicate a more complex situation than anticipated. Thus the high PGM to PHRL ratios found in most of the white tracts could mean that G1P has another use in these tracts, perhaps in the formation of uridine diphosphoglucose.

SUMMARY

1. New micromethods are presented for measuring hexokinase, phosphoglucoisomerase, phosphofructokinase, glycogen phosphorylase, and phosphoglucomutase in 1 or 2 γ of tissue (dry weight). The methods have been validated for use with frozen-dried brain that is either fresh or stored at $-20°$ for several years.

2. The new methods, together with existing procedures of similar sensitivity for aldolase and glucose-6-phosphate dehydrogenase, have been used to measure the distribution of seven major enzymes of hexose metabolism among five white tracts, two molecular layers, two layers of cell bodies, and a layer of packed dendrites from rabbit brain.

3. Cell body layers (of cerebellum and Ammon's horn) tend to be lower in content than other gray areas with respect to every enzyme measured. White tracts vary greatly among themselves. Heavily myelinated tracts are low in hexokinase activity and rich in glucose-6-phosphate dehydrogenase. In respect to the other five enzymes, all of the tracts examined are about as active on a fat-free dry weight basis as the cell body layers.

4. Enzyme members of the same metabolic path tend to vary in a similar manner, but the correspondence is by no means perfect.

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CATALYTIC AND ADSORPTIVE PROPERTIES OF TESTICULAR MICROSOMES*

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(Received for publication, September 9, 1957)

In the course of attempts to purify the enzymes found in the testicular microsomes which oxidize progesterone to testosterone (1–3), it was observed that these enzymes are active only in their particular state. Thus, a 100,000 × *g* supernatant fluid which exhibited only traces of steroid-oxidizing activity could be made active by precipitation of the proteins either by dialysis or salt. Both procedures resulted in the formation of an insoluble precipitate that contained the enzymatic activity.

When it was observed that these "generated" particles, as well as microsomes, can almost quantitatively adsorb steroids in an aqueous solution, the hypothesis was formulated that these particles were templates on which substrates and enzymes could come into contact. This prompted a survey of the adsorptive and catalytic properties of testicular microsomes.

EXPERIMENTAL

Particles were prepared by homogenization of decapsulated guinea pig testicles in cold 0.03 M Tris[1] (pH 7). The large particles were removed by centrifugation at 10,000 × *g* for 20 minutes, and then the microsomes were collected by centrifugation of the supernatant fluid at 100,000 × *g* for 20 minutes. The microsomes were then washed several times either with more buffer or with 1 M NaCl, and finally resuspended by homogenization in sufficient buffer to give an optical density of 1.0 at 280 mμ with a light path of 1 cm. The emulsion thus obtained was stable for 1 hour.

Studies of adsorption were performed by adding 1 ml. of the microsomal suspension to 9 ml. of Tris buffer, containing varying amounts of substrates. This mixture was stirred gently for 10 minutes at 0°, and

* Supported by the United States Public Health Service, grant No. RG4758.

† Markle Scholar in Medical Sciences.

[1] The following abbreviations are used in the text: Tris, tris(hydroxymethyl)-aminomethane; DPNH, reduced diphosphopyridine nucleotide; RNA, ribonucleic acid; TPNH, reduced triphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; FMN, flavin mononucleotide; and FAD, flavin adenine dinucleotide.

was then centrifuged at 100,000 × *g* for 20 minutes (Spinco model L refrigerated centrifuge). The amounts of substrate in the supernatant fluid and microsomes were then determined. Control experiments without substrate were run in every case.

Proteins were assayed by the spectrophotometric method of Warburg and Christian (4), and in some instances by the method of Lowry *et al.* (5). Steroids, amino acids, phosphate, sulfate, lactate, acetate, and butyrate were radioactive, and were assayed by gas flow counting techniques. The nucleotides, purine bases, and coenzymes were assayed spectrophotometrically; glucose 6-phosphate was assayed enzymatically (6), glucose (7), molybdate (8), cobalt (9), and magnesium (10) were determined by colorimetric procedures, and iron (as the ferrous *o*-phenanthroline complex (11)) was determined after reduction by testicular microsomes and DPNH (12).[1] Sodium and potassium were measured with the flame photometer.

The various enzymes were assayed as follows: lactic dehydrogenase[2] by the method of Kubowitz and Ott (13), malic dehydrogenase[2] by the method of Mehler *et al.* (14), alcohol dehydrogenase[2] by the procedure described by Theorell and Bonnichsen (15), xanthine oxidase[2] by the optical method of Kalckar (16), and glucose-6-phosphate dehydrogenase by the method of Warburg and Christian (6). To eliminate the errors due to light scattering effects in these particulate suspensions, frequent base lines and complete spectra were obtained with the use of the Beckman model DK-2 recording spectrophotometer.

Microsomal Constituents—Phosphate and nitrogen were assayed on a digest (sulfuric acid and hydrogen peroxide) of dried microsomes by the methods of Fiske and Subbarow (17) and of Brown *et al.* (18), respectively. Lipide was measured gravimetrically after alcoholic-ether extraction (19); RNA was approximated by use of the orcinol color test and the 260:280 mμ ratios; porphyrin was determined by its Soret band; and the flavins were measured fluorometrically (20). Flavins were also assumed to be present in the microsomes because of the presence of large amounts of the DPNH and TPNH cytochrome *c* reductases in these microsomes (11).

Results

The constituents of these testicular microsomes appear to differ in several respects from those of other microsomes isolated from various organs. The low content of protein and fat may result from the several

[2] Yeast hexokinase, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, and malic dehydrogenase were obtained from the Sigma Chemical Company. Lactic dehydrogenase (twice crystallized) was a gift from Dr. George W. Schwert, Jr. Xanthine oxidase was obtained from the Worthington Biochemical Corporation.

washes which were performed before analysis. The high phosphate value may be spurious in that, as demonstrated in Fig. 3, these microsomes have a marked affinity for phosphate, and the high phosphate content may be due to adsorption rather than the RNA or the phospholipide content. The absence of an appreciable amount of porphyrin distinguishes them from liver microsomes, which contain a cytochrome *b* (21).

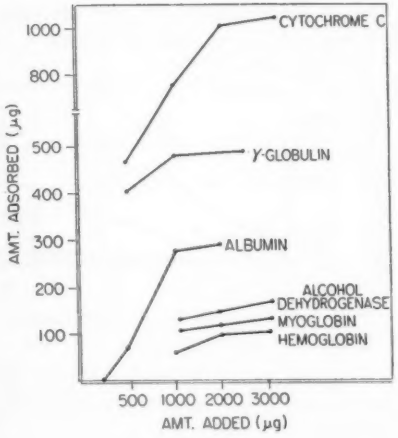

FIG. 1

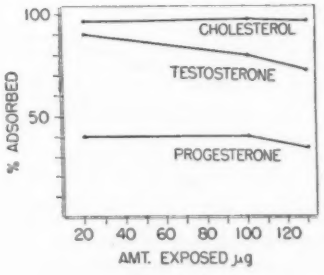

FIG. 2

FIG. 1. Adsorption of protein by testicular microsomes. Adsorption was performed by suspending 1 ml. of the microsomal suspension in 10 ml. of Tris buffer (pH 7) which contained the protein to be adsorbed. The mixture was stirred at 0° for 10 minutes, and the microsomes were resedimented at 100,000 × *g*. Protein was then assayed in the supernatant fluid by the method of Lowry *et al.* (5). A control experiment without added protein was run in each case. Alcohol dehydrogenase was assayed enzymatically both in the microsomes and in the supernatant fluid.

FIG. 2. Adsorption of steroids by testicular microsomes. The conditions were the same as in Fig. 1. The isotopic steroids were assayed by counting the aliquots in a gas flow counter.

The following values were obtained: 15 per cent protein, 18 per cent nitrogen, 3 per cent lipide, 20 per cent phosphate, and appreciable amounts of RNA, iron, and flavin.

Adsorption Studies—Figs. 1 through 4 show the adsorption of the various types of compounds by these microsomes. Substances which are adsorbed include proteins, steroids, phosphate, and iron. Of the proteins, the adsorption of cytochrome *c* and γ-globulins is most striking; however, such enzymes as alcohol dehydrogenase and lactic dehydrogenase are also adsorbed. The marked adsorption of phosphate remains

unexplained, but is probably not due to its charge because other small anions such as sulfate and acetate are not adsorbed. Once the phosphate is adsorbed, it is difficult to wash it away, and preliminary data indicate that it no longer exists as free inorganic phosphate. (These data will be presented in another report.) Substances showing less than 5 per cent adsorption include nucleic acids, nucleotides (ATP, ADP, AMP, GTP, GDP, GMP, TPN, DPN, TPNH, and DPNH), glucose, glucose 6-phosphate, amino acids (phenylalanine, glycine, and glutamic acid), flavins (FMN, FAD, and riboflavin), thiamine, and guanosine.

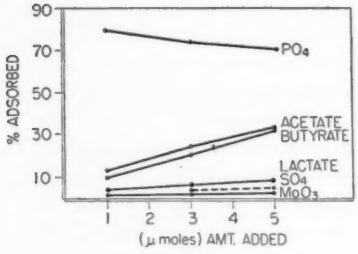

FIG. 3

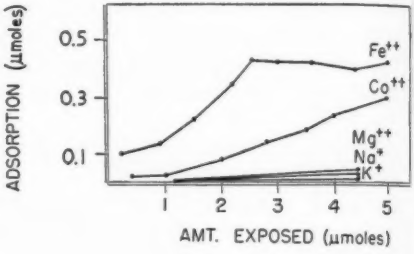

FIG. 4

FIG. 3. Adsorption of anions by testicular microsomes. The conditions were the same as in Fig. 1. Phosphate, acetate, lactate, and butyrate were assayed isotopically, and sulfate and molybdate were determined colorimetrically.

FIG. 4. Adsorption of metallic ions by testicular microsomes. The conditions were the same as in Fig. 1. Fe^{++}, Co^{++}, and Mg^{++} were measured colorimetrically, and Na^{+} and K^{+} by flame photometry.

Littlefield and Keller (22) reported that liver microsomes adsorb sodium and potassium, but these testicular microsomes exhibit no adsorption of these cations. However, iron is adsorbed.

Catalytic Properties—Since these microsomes adsorb proteins, it is to be expected that they should affect enzyme rates. Indeed, there are many observations which indicate that insoluble surfaces and complex molecules do markedly affect these rates. Thus, Keilin and Hartree (23) noted the activation of succinic dehydrogenase in the presence of calcium phosphate gel. McLaren (24) has observed a difference in the pH optimum of soluble enzymes *versus* that of particulate enzymes. Dellert and Strarmann (25) have noted a marked increase in the activity of trypsin in the presence of the insoluble polymer, polylysine. The presence of the increasing concentration of small ions (NaCl) has been shown to increase the rate of catalysis by lactic dehydrogenase (26, 27). In Figs. 5 and 6 is observed the marked stimulation of the initial rates of lactic and malic dehydrogenases in the presence of these microsomes. Before being washed,

they contained large amounts of both malic dehydrogenase and lactic dehydrogenase, and, even after as many as four washes with a solution of 1 M NaCl or buffer, a small fraction of enzymatic activity remained.

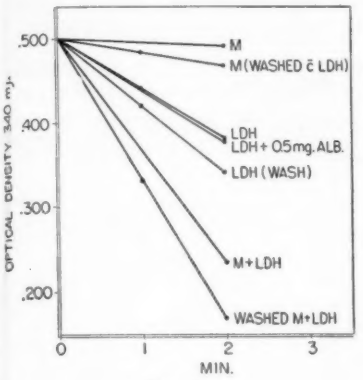

FIG. 5

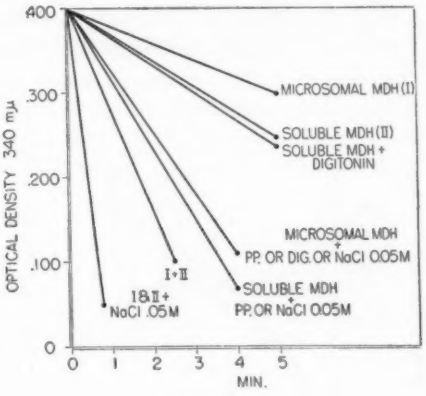

FIG. 6

FIG. 5. Stimulation of the catalytic rate of lactic dehydrogenase by testicular microsomes. The incubation was carried out at room temperature, at pH 7 (0.1 M Tris buffer). The enzyme was 0.5 ml. of microsomes (M) washed four times with 1 M NaCl (30,000 to 100,000 × *g*). The assay was performed with a No. DK-1 recording spectrophotometer. The blank cuvette contained all of the components except DPNH. Microsomes were exposed to lactic dehydrogenase (LDH) for 10 minutes at 0°, and then resedimented at 100,000 × *g*. The supernatant fluid (LDH wash) and microsomes (washed with LDH) were assayed for lactic dehydrogenase. All assays (7) were performed in Beckman cuvettes, and crystalline lactic dehydrogenase was used. Blanks without either pyruvate or DPNH or both were run in all experiments. These particles contained no DPNH oxidase. Alb. = albumin.

FIG. 6. Stimulation of the catalytic activity of malic dehydrogenase (MDH) by testicular microsomes, digitonin, and NaCl. Incubations were performed at room temperature, at pH 7 (Tris 0.02 M) by the method of Mehler *et al.* (14). Microsomal MDH, 0.5 ml. of the standard solution of microsomes (washed once with NaCl (1 M)). Soluble MDH, commercial malic dehydrogenase. Assays were performed on a recording spectrophotometer. Dig., digitonin, 0.05 M; PP, sodium pyrophosphate, 0.05 M. I + II = soluble malic dehydrogenase + microsomal malic dehydrogenase.

However, after a known amount of either dehydrogenase was added to the microsomes, the net activity was more than 100 per cent greater than that of the added fraction.

The fact that microsomes contain these two dehydrogenases suggested the possibility that the presence of added enzymes caused leaching of the microsomal enzymes, with the stimulatory effect resulting simply from the presence of more enzyme in solution. This possibility was eliminated by

the data in Fig. 5. The microsomes were exposed to known amounts of lactic dehydrogenase, were resedimented, and the enzyme was assayed in the resultant supernatant fluid as well as in the microsomes. There was no increase in the activity of the enzyme in the supernatant fluid. Furthermore, when these same lactic dehydrogenase-exposed microsomes were again added to more lactic dehydrogenase, their stimulatory capacity was

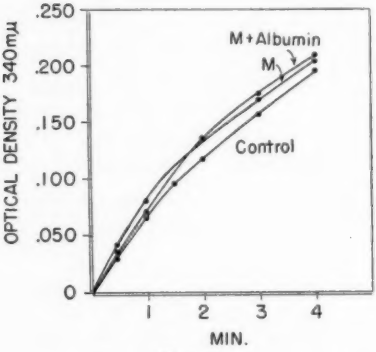

FIG. 7

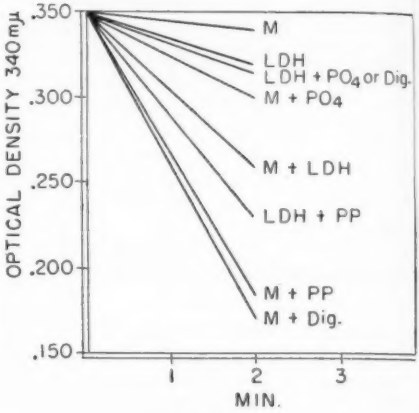

FIG. 8

FIG. 7. Effect of microsomes on alcohol dehydrogenase. Incubations were performed at room temperature by the method of Theorell (0.02 M Tris, pH 7.0). M, 0.5 ml. of standard washed testicular microsomal suspension. Albumin had no effect in the amounts of 0.1 to 5 mg. per 3 ml. Blanks were run as in Fig. 5.

FIG. 8. Stimulation of catalytic activity of lactic dehydrogenase by microsomes, anions, and digitonin. Conditions were the same as in Fig. 5. M, 0.5 ml. of salt-washed microsomes; LDH, crystalline lactic dehydrogenase; Dig., 0.05 M digitonin; PP, 0.05 M sodium pyrophosphate (pH 7.0); PO_4, 0.05 M sodium phosphate (pH 7.0). Blanks were run as in Fig. 5.

still equally effective, thereby indicating that the recentrifugation had not resulted in the removal of their stimulatory activity.

Fig. 7 indicates the specificity of the stimulatory effect of microsomes. There was little effect from these microsomes on the rates of action of soluble alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, hexokinase, or xanthine oxidase. The only other enzyme, thus far tested, whose activity was enhanced was catalase. However, this effect is somewhat uncertain, because all of the catalase activity of the microsomes themselves could not be removed.

Mechanism of Microsomal Catalysis—Hakala (26) and Nygaard and Theorell (27) have observed that the enzymatic activity of lactic dehydro-

genase is enhanced by increasing the concentrations of sodium chloride, and Figs. 6 and 8 show that this salt effect also occurs with malic dehydrogenase. Moreover, it has been noted that many other salts can be substituted, *e.g.* potassium chloride, sodium sulfate, or sodium pyrophosphate. Sodium phosphate, however, was either ineffective or inhibitory at high concentrations. These effects of salts were also demonstrable with the lactic and malic dehydrogenases of the microsomes. The possibility arose that the stimulatory effect of the microsomes results from the leaching of salt from the microsomes into the medium. This did not seem to be the case, however, because the effects of microsomes and salts were

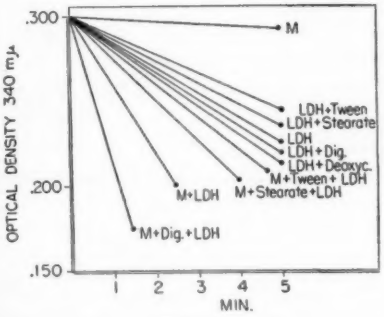

FIG. 9

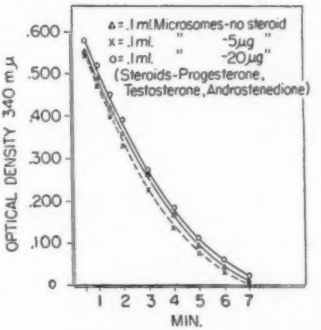

FIG. 10

FIG. 9. Effect of detergents on microsomal LDH and soluble lactic dehydrogenase. Amount of detergents added, 2 μmoles. The assay was performed as in Fig. 6. Enzyme, testicular microsomes washed once with 1 M NaCl (0.5 ml.). Dig., digitonin; Deoxyc., sodium deoxycholate. Blanks were run as in Fig. 5.

FIG. 10. Effect of steroids on microsomal lactic dehydrogenase. The assay was performed as in Fig. 6. Steroids were added in 0.01 ml. of alcohol.

additive. Moreover, the repeated washing of these microsomes with buffer did not lessen their catalytic properties. Further, when the microsomes were resedimented in the presence of lactic dehydrogenase, the enzyme that remained in the supernatant fluid was no longer stimulated (Fig. 8).

Since these microsomes are fat-containing and insoluble, surface-active agents should have some effect on their catalytic properties. Fig. 9 shows that Tween 80, sodium stearate, and sodium deoxycholate inhibit the catalytic activity of microsomal lactic dehydrogenase, whereas digitonin stimulates this enzymatic activity. Steroids, on the other hand, had no effect (Fig. 10). Amphenone B has also been noted to be inhibitory (12). The stimulatory effect of digitonin could result from the leaching out of "bound-inactive" malic dehydrogenase. However, Fig. 11 demonstrates

that this is not the case, because, in the presence of 10^{-3} M digitonin, the microsomes could still be sedimented, and the supernatant fluid from this sedimentation did not increase the enzymatic activity.

An effort was made to ascertain which constituents of the microsomal surface are necessary for this stimulating activity. Microsomes were digested at 30° for 30 minutes with pancreatic lipase, trypsin, or ribonuclease, and were resedimented (Fig. 12). The extent to which material

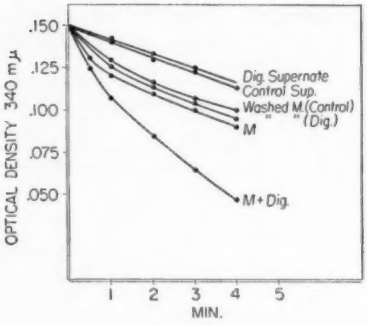

FIG. 11

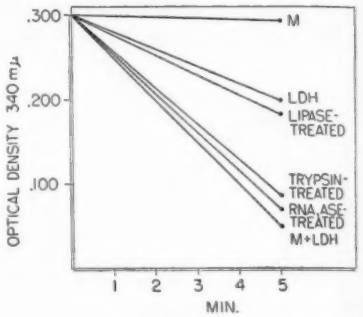

FIG. 12

FIG. 11. Effect of digitonin exposure on microsomal malic dehydrogenase. The assay was performed as in Fig. 5. 0.3 ml. of microsomes (washed once with NaCl) was exposed to digitonin 2×10^{-3} M at 0° for 10 minutes, then resedimented at 100,000 × *g*. Microsomes (washed M Dig.) and supernatant fluid (Dig., digitonin supernatant) were assayed, along with controls. Blanks were run as in Fig. 5.

FIG. 12. Effects of hydrolytic enzymes on testicular microsomes. 0.5 ml. aliquots of microsomes (washed twice with NaCl) were incubated with 1 mg. of either lipase (commercial), trypsin (crystalline), or RNAase (commercial) for 30 minutes at 30°, pH 7, and then resedimented. Each batch of particles was tested for its ability to stimulate crystalline lactic dehydrogenase, under the same conditions as in Fig. 6. Digestion of the microsomes was followed by the measurement of the increase in absorption at 260 and 280 mμ in the supernatant fluid. Blanks were run as in Fig. 5.

was extracted was estimated by the measurement of the increase in absorption at 260 and 280 mμ in the supernatant fluid. Only the lipase-treated microsomes exhibited appreciable liberation of ultraviolet-absorbing material. Lipase was also the only enzyme which inactivated the stimulatory activity of microsomes on lactic dehydrogenase. Leaching of material absorbing at 260 to 280 mμ was obtained after digestion by trypsin and ribonuclease if the particles were first digested with lipase, and this suggests that the surface of these particles is fatty in nature and that only after removal of this fatty surface do trypsin and ribonuclease have access to their substrates within the microsomes.

DISCUSSION

It has been demonstrated that intracellular surfaces, *i.e.* microsomes, possess specific adsorptive properties, and specific effects on rates of enzymatic activity. It should be noted, however, that the only known enzymes whose rates of catalysis were accelerated by these microsomes were those which are normally found in microsomes. This may mean that these particles have specific sites for specific enzymes and that only when these sites are filled do the enzymes exhibit a greater efficiency in their catalytic roles.

No kinetic treatment of this type of enhanced catalytic activity has been undertaken. However, the Michaelis constant for pyruvate, obtained on the lactic dehydrogenase in the presence and the absence of microsomes, seemed to be the same (about 5×10^{-5} M, pH 7, at 24°).

Since these testicular microsomal particles have been shown to adsorb fats and proteins and because, as far as is known, many of the enzymes that have been found associated with microsomes are concerned either with insoluble substrates, *e.g.* steroids (1, 2, 28) and drugs (29), or large polymers, *e.g.* proteins (30), this structure may serve as a catalyst or template on which water-soluble enzymes and other insoluble substrates can come into contact. Since these surfaces can affect rates of enzymatic activity and are presumed to exist inside the cell (31), any substance which can affect such surfaces, *e.g.* insoluble materials such as many of the hormones, might exert some control over the enzymatic activity within the cell.

SUMMARY

Testicular microsomes possess specific adsorptive properties as well as specific effects on rates of enzymatic activity.

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EFFECTS OF AMPHENONE B ON THE ENZYMATIC PROPERTIES OF TESTICULAR MICROSOMES*

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(Received for publication, September 9, 1957)

Amphenone B[1] has been shown to have widespread biological effects; *e.g.*, steroid production is blocked in the adrenal (1), I^{131} uptake is blocked in the thyroid gland (2), progestational activity is induced in the Clauberg rabbit (3), and anesthetic effects have been observed in various animals (4). The adrenal perfusion experiments of Rosenfeld and Bascom (1) indicate that steroidogenesis is blocked at a drug concentration that does not impair the oxygen consumption of the intact adrenal gland. Each of these observations is compatible with inhibition of an oxidative pathway, *e.g.* $I^- \rightarrow I^0$, or hydroxylation of steroids, by a drug which does not affect the normal cytochromal respiratory systems. Since there are no reports on the effects of Amphenone B in the liver microsomal TPNH[2] oxidase systems (5) or in the steroid TPNH oxidase systems found in various glands (6, 7), it was thought worth while to investigate the effect of this drug on isolated microsomal systems from guinea pig testes, since progesterone is oxidized to testosterone in these systems (8, 9). The present report demonstrates that this microsomal steroid-oxidizing system is blocked by Amphenone B, and some observations concerning the mechanism of this inhibition are also reported.

EXPERIMENTAL

The microsomes were prepared by homogenizing mature guinea pig testicles in 0.02 M Tris buffer (pH 7), centrifuging the large particles at 12,000 × *g* for 10 minutes, decanting the supernatant fluid, and centrifuging the small particles at 100,000 × *g* for 20 minutes. These particles were then resuspended in ice-cold 1 M NaCl and again sedimented at 100,000 × *g* for 20 minutes. The microsomes were then resuspended in 0.02 M Tris (4 ml. per testicle).

* Supported by the United States Public Health Service, grant No. RG4758.

† Markle Scholar in Medical Sciences.

[1] Amphenone B (1,2-bis(*p*-aminophenyl)-2-methyl-1-propanone dihydrochloride) was obtained from Dr. Robert Gaunt, Ciba Pharmaceutical Products, Inc.

[2] The following abbreviations are used in the text: TPNH, reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane; and DPNH, reduced diphosphopyridine nucleotide.

The steroids and Amphenone B were isolated by precipitation of the incubation mixture with 2 parts of ethanol. 2 parts of water were then added, and the Amphenone B and steroids were removed by extractions with ether. The ether was washed with dilute Na_2CO_3. The steroids were separated by paper chromatography (Zaffaroni and Burton (10), Bush (11), and Neher and Wettstein (12)) (and assayed at 240 mμ) as well as by their radioactivity. Inasmuch as these microsomes contain no 3-keto reductases, quantitative recoveries of all the steroids were possible with this one assay method. Since Amphenone B also absorbs strongly at 240 mμ and is easily separated in the Zaffaroni paper chromatogram, it could be isolated and assayed in the same manner. Acetate was isolated after its distillation and separated as the ammonium salt in a *tert*-butanol-NH_4OH paper chromatographic system (13) and assayed by its C^{14} content.

Commercial progesterone-4-C^{14} and progesterone-21-C^{14} were used as substrates. Oxygen consumption was measured by the conventional Warburg technique.

TPNH was assayed by two different methods. Since the microsomal suspensions used in the incubations were too opaque to utilize the standard 340 mμ assay, and since these microsomes catalyze the quantitative reduction of both 2,6-dichlorophenol-indophenol and ferric iron in the presence of TPNH, the time curves describing the disappearance of the dye (610 mμ) and the appearance of ferrous *o*-phenanthroline (510 mμ) could be obtained (14). Known amounts of TPNH gave straight line curves with both assay methods.

Malic dehydrogenase was assayed according to the method of Mehler and coworkers (15), lactic dehydrogenase by the procedure of Kubowitz and Ott (16), and catalase activity by the method of Bonnichsen *et al.* (17). Since lactic and malic dehydrogenases were assayed in particulate suspensions, the Beckman model DK-1 recording spectrophotometer was used, and frequent base lines were obtained.

Results

Inhibition of Microsomal Enzymes—Table I indicates that Amphenone B inhibits the hydroxylation of progesterone, the oxidative cleavage of 17α-hydroxyprogesterone, and the reduction of androstenedione by testicular microsomes.

Table II indicates that these testicular microsomes contain a TPNH oxidase system (DPNH is inactive) and that this TPNH oxidase is blocked by Amphenone B and testosterone. A soluble TPNH oxidase has also been observed in the supernatant fluid (after centrifugation at 100,000 × *g*) from these testicles. This soluble TPNH oxidase is partially purified by use of hydroxyapatite (18) and protamine sulfate, but it is not

affected by 10^{-3} M Amphenone B. Table II further shows that the two steroids which are oxidized by these microsomes do not block the TPNH oxidase, as does the enzymatically inert steroid, testosterone.

Figs. 1 and 2 demonstrate that other microsomal enzymes, *i.e.* lactic dehydrogenase and malic dehydrogenase, are also blocked. However,

TABLE I

Steroid Oxidation and Amphenone B

Experiment No.	Substrate	Enzyme (1.5 ml.)	Amphenone B	Formation		
				17α-Hydroxyprogesterone	Testosterone	Acetic acid
				per cent	*per cent*	*per cent*
1	Progesterone-21-C^{14}, 0.5 μmole	Microsomes	0	15	83	85
2	" 0.5 "	"	10^{-3} M	5	0	0
3	" 0.5 "	"	10^{-4} "	10	15	20
4	17α-Hydroxyprogesterone, 0.5 μmole	"	0		25	
5	17α-Hydroxyprogesterone, 0.5 μmole	"	10^{-3} M		0	
6	17α-Hydroxyprogesterone, 0.5 μmole	"	10^{-4} "		10	
7	Androstenedione, 0.5 μmole	"	0		95	
8	" 0.5 "	"	10^{-3} M		15	
9	" 0.5 "	Soluble enzyme	0		75	
10	" 0.5 "	Soluble enzyme	10^{-3} M		73	

Each flask contained, in addition to the substances listed, 0.3 μmole of TPN, 5 μmoles of glucose 6-phosphate, NaCN, 10^{-4} M, and sufficient glucose-6-phosphate dehydrogenase (obtained from the Sigma Chemical Company) to reduce all the added TPN in 1 minute. The microsomes were the 30,000 to 100,000 × *g* particles obtained from testicular homogenate (10 ml. of 0.02 M Tris-HCl buffer per three guinea pig testicles). The soluble enzyme was the 100,000 × *g* supernatant fluid. Incubations were performed in closed flasks at 30° for 1 hour with O_2 as the gas phase. The enzymatic products were assayed as described in the text.

these two enzymes are not inhibited in the soluble forms present in the testicular supernatant fluid, and the microsomal enzymes are not inhibited when incubated in Tris buffer at pH 5.8. The incubations were performed at this pH value because the basic Amphenone B at a concentration of 10^{-3} M is soluble in water at this pH. Fig. 5 indicates that, at pH 5.8, Amphenone B is not adsorbed to microsomes, whereas at pH 7.0 it is. Fig. 2 further demonstrates that this Amphenone B inhibition of malic dehydrogenase persists after the microsomes have been resedimented and

suspended in buffer which does not contain Amphenone B. Since the supernatant fluid from these microsomes exposed to Amphenone B did not contain any excess malic dehydrogenase activity, it is not likely that the Amphenone B acted by dissolving the malic dehydrogenase within the microsomes.

TABLE II

Inhibition of Testicular Microsomal TPNH Oxidase by Amphenone B and Testosterone

Experiment No.	Enzyme	Inhibitor	TPNH	O_2 used	TPNH used	Substrate used
			μmole	*μmole*	*μmole*	
1	MTO*	†	0	0.0		
2	"	†	0.5	0.22	0.34	
3	"	Amphenone B, 10^{-3} M	0.5	0.0	0.06	
4	"	Testosterone, 0.5 μmole	0.5	0.1	0.15	
5	"	Progesterone, 0.5 μmole	0.5	0.41	0.5	0.31†
6	"	17α-Hydroxyprogesterone, 0.5 μmole	0.5	0.27	0.42	0.22‡
7	"	Androstenedione, 0.5 μmole	0.5	0.14	0.36	0.3‡
8	"	N_2	0.5	0.1	0.1	

Each flask contained 2 ml. of enzyme and NaCN, 10^{-4} M, in addition to the substances listed. Incubations were made under air or N_2 at 30° in 0.02 M Tris-HCl buffer. TPNH was assayed at the end of and during incubation on aliquots of the reaction mixture by 2,6-dichlorophenol-indophenol reduction and by iron reduction.

* MTO = testicular microsomes washed with 1 M NaCl.

† Of the 0.31 μmole of progesterone oxidized, 70 per cent was converted to 17α-hydroxyprogesterone and 20 per cent to testosterone.

‡ The steroid product was 100 per cent testosterone.

Rate curves were also obtained with other pyridine nucleotide-dependent enzymes in the microsomes; *i.e.*, DPNH and TPNH cytochrome *c* reductases, and again the inhibition by Amphenone B was observed (Fig. 3). However, no inhibition of microsomal iron reductase or 2,6-dichlorophenol-indophenol diaphorase was observed, whereas catalase and ascorbic acid oxidase were inhibited (Fig. 4). Catalase activity was measured manometrically and by titration with permanganate. Ascorbic acid oxidase was measured only manometrically.

Inhibition of Adsorption by Amphenone B—Previously it had been shown that testicular microsomes have rather specific and marked adsorptive

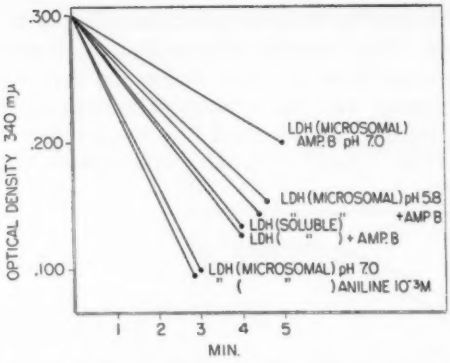

FIG. 1. Inhibition of microsomal lactic dehydrogenase by Amphenone B. Incubations were carried out at room temperature in Beckman cuvettes, 1 cm. light path, at pH 7 (Tris-HCl, 0.1 M). LDH (microsomal), testicular microsomes washed twice with 1 M NaCl, obtained from the same guinea pig testicle (0.3 ml. of a suspension whose optical density was 1.0 at 280 mμ) (18). LDH (soluble), crystalline lactic dehydrogenase obtained from Dr. George Schwert, Jr. Amphenone B (AMP-B) was 10^{-3} M in all incubations. (Blanks, without sodium pyruvate, were run in each experiment. These blanks indicated that these microsomes possess no DPNH oxidase activity.)

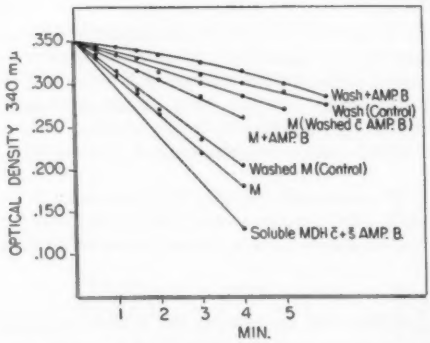

FIG. 2. Microsomal malic dehydrogenase after exposure to Amphenone B. The conditions are the same as in Fig. 1. The microsomes were exposed to 10^{-3} M Amphenone B (AMP-B) for 10 minutes at 0°, and then resedimented. Both the microsomes (M washed with Amp-B) and the 100,000 × *g* supernatant fluid (wash + Amphenone B) were assayed for the enzyme. Soluble MDH, malic dehydrogenase obtained from the Sigma Chemical Company. The enzyme was 0.3 ml. of testicular microsomes washed with 1 M NaCl. Amphenone B, 10^{-3} M, was used in all incubations. (Blanks, without sodium pyruvate, indicate that these microsomes have no DPNH oxidase activity.)

properties and that, in low concentration, steroids may be almost quantitatively adsorbed. Figs. 5 and 6 indicate that Amphenone B is also adsorbed to these microsomes to a high degree and that, when this drug is adsorbed, it interferes with the subsequent adsorption of steroids (18). Aniline is not adsorbed, however, and does not interfere with the microsomal enzymes (Figs. 1 and 5). In these experiments, Amphenone B was

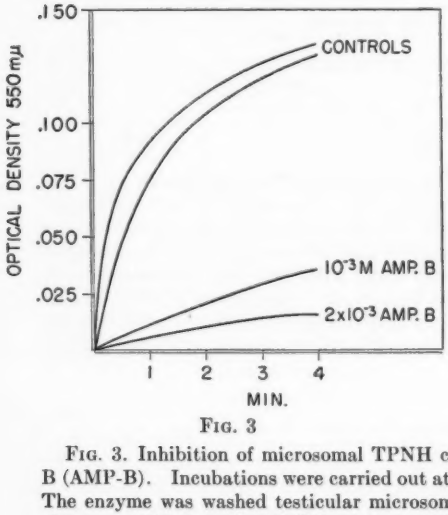

FIG. 3

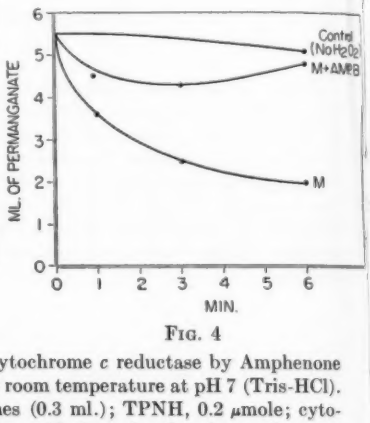

FIG. 4

FIG. 3. Inhibition of microsomal TPNH cytochrome *c* reductase by Amphenone B (AMP-B). Incubations were carried out at room temperature at pH 7 (Tris-HCl). The enzyme was washed testicular microsomes (0.3 ml.); TPNH, 0.2 μmole; cytochrome *c*, 0.0002 μmole; NaCN, 10^{-4} M. (The oxidation of reduced cytochrome *c* was completely inhibited by the cyanide in this system.) These microsomes also contained a DPNH cytochrome *c* reductase which was equally inhibited by Amphenone B.

FIG. 4. Inhibition of microsomal catalase by Amphenone B (AMP-B). Incubations were carried out at room temperature at pH 7; Tris buffer, at pH 7.0. The enzyme was 0.1 ml. of washed microsomes in a final volume of 3 ml. Amphenone B, 10^{-3} M; H_2O_2, 60 μmoles.

assayed after its isolation by measuring its intense absorption at 240 mμ. The entire ultraviolet spectrum was examined in each experiment to eliminate the possibility that ultraviolet-absorbing material might be leaching from the microsomes, and thus obscuring our results. In no case, however, was there any skewing of the Amphenone B spectrum. The adsorption measurements were made as previously described (18).

Since Amphenone B interferes with the adsorption and oxidation of steroids by microsomes and since those microsomal enzymes which use DPNH or TPNH are also blocked by Amphenone B, it was thought that

Amphenone B might be similarly inhibiting the adsorption to the microsomes of these coenzymes. However, this could not be demonstrated

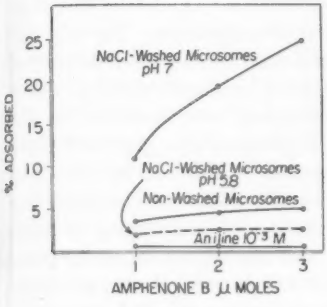

FIG. 5

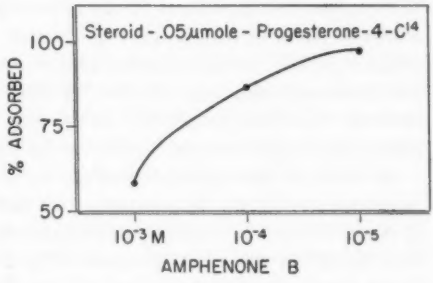

FIG. 6

FIG. 5. Adsorption of Amphenone B by testicular microsomes. Adsorption studies were performed as previously described (18). Aniline and Amphenone B were assayed in the supernatant fluid on a recording spectrophotometer by measuring their intense absorption at 240 mμ. Aniline was not adsorbed to either washed or non-washed microsomes.

FIG. 6. Inhibition of microsomal steroid adsorption by Amphenone B. 0.3 ml. of microsomes (washed once with 1 M NaCl) (optical density 1.0 at 280 mμ) was exposed to the Amphenone B for 10 minutes, with stirring, before addition of the steroid. The steroid was assayed (after centrifugation at 100,000 × *g*) in the microsomes and in the supernatant fluids by its C^{14} content.

TABLE III

Oxygen Tension and Amphenone B Inhibition of Microsomal TPNH Oxidase

	TPNH used
	μmole
Control (air)	0.5
Amphenone B, 10^{-3} M + air	0
" " 10^{-4} " + "	0.15
" " 10^{-3} M + 100% O_2	0.1
" " 10^{-4} M + 100% "	0.40
Control (N_2)	0.02

The enzyme used was 1 ml. of testicular microsomes washed with 1 M NaCl; incubation was for 30 minutes at 30° in Tris (0.1 M), pH 7. TPNH was assayed by titrating with 2,6-dichlorophenol-indophenol at the end of the incubation period. Each flask contained 0.5 μmole of TPNH, and NaCN, 10^{-4} M, in addition to the substances listed, in a final volume of 1.2 ml.

since neither DPNH nor TPNH is bound to microsomes in detectable amounts in the presence or the absence of Amphenone B (18).

An alternative explanation of the TPNH oxidase effects would be the inability of O_2 to come into contact with the Amphenone B-coated microsomes. Table III indicates that the Amphenone B inhibition can be partially overcome by increasing the oxygen tension. However, this does not explain the inhibition of the anaerobic dehydrogenases. Further, the catalytic rates of soluble glucose oxidase and xanthine oxidase, neither of which is present in the microsomes, are unaffected by Amphenone B. Since these steroid oxidations require TPNH, some data were collected on the systems which supply TPNH in the cell. Neither soluble glucose-6-phosphate dehydrogenase nor isocitric dehydrogenase is inhibited by this drug.

In none of the systems studied, including the complete testicular homogenate, could we demonstrate any metabolism of Amphenone B itself. It could always be isolated quantitatively and chromatographically pure. This probably rules out the possibility that Amphenone B acts competitively as an electron drain on the steroid-oxidizing system of the cell.

DISCUSSION

Since it has been demonstrated that Amphenone B has rather widespread endocrinological effects *in vivo*, one might expect its blocking effects to be related to some fundamental system which endocrine organs have in common. However, since, at least in the adrenal gland, it has no effect on the major respiratory path but does block the oxidation of steroids by enzymes which require TPNH and O_2, its action must be related in some way to the parts of the cells which use this more specific oxidative pathway. This paper would indicate that the blocking action of Amphenone B requires the presence of cell structure, *i.e.* microsomes, because the enzymes thus far tested are inhibited only when they are contained within the microsomal structure. The marked inhibition of the microsomal TPNH-specific oxidase may well explain why this drug prevents the oxidation of the steroids because steroid oxidation in the testes can be accomplished only when the TPNH is being oxidized simultaneously. Since data from this laboratory indicate that the TPNH and oxygen are needed in stoichiometric amounts (9), it would appear that the actual oxidant is either hydrogen peroxide or a hydroxyl radical. Thus, when the generating system, TPNH oxidase, is blocked, no oxidation of the steroids can occur.

The fact that Amphenone B also blocks the ability of the microsomes to adsorb the steroids could also explain the inhibition of their oxidative as well as their reductive enzymes, in that the steroid substrate would be unable to penetrate to its enzyme within the microsome.

Repeated attempts to render these steroidal microsomal enzymes soluble have failed; therefore, the effects of Amphenone B on soluble steroid hydroxylases have not been studied. However, androstenedione reductase,

as well as the 17α-hydroxy, 20-keto reductases (which are present in the soluble 100,000 × *g* supernatant fluid) (9), are not inhibited by Amphenone B (Table I).

The only microsomal enzymes thus far tested which are not inhibited by Amphenone B are the diaphorases and the iron reductases. Since these diaphorases can be readily removed from the microsomes by washing, perhaps these enzymes are always in the "soluble form," thus accounting for the inability of the Amphenone B to block their action. A similar statement can be made for the microsomal iron reductase; Weber *et al.* (14) have shown that any flavin, free or protein-bound, will act as an iron reductase, and Amphenone B does not inhibit this type of reduction of soluble iron by non-protein systems.

The sum of these data suggests that Amphenone B is a general microsomal poison. This toxic effect is probably related to the marked affinity of microsomes for Amphenone B at pH 7 which may be related to the strong basicity and water insolubility of the Amphenone B. Other basic materials, such as cytochrome *c* (18), and many water-insoluble substances are also strongly adsorbed to the microsomal surface. However, aniline, despite its basicity and low solubility, is not adsorbed by these microsomes, and it does not inhibit (at 10^{-3} M) the microsomal oxidases or pyridine nucleotide reductases.

From the previous observation that microsomes can affect the rates of reactions catalyzed by soluble enzymes, plus the fact that substances like Amphenone B and testosterone can inhibit intramicrosomal enzymes, a simple generalization appears: intracellular metabolic rates can be controlled by intracellular surfaces and architecture; furthermore, substances that alter the character of these surfaces, by adsorption or otherwise, can interfere with this function of these surfaces.

SUMMARY

Amphenone B inhibits the catalytic activities of many enzymes in testicular microsomes. However, if these enzymes are in the soluble state, no inhibition by Amphenone B can be demonstrated.

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THE CONVERSION OF PROGESTERONE TO ANDROGENS BY TESTES*

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(Received for publication, November 18, 1957)

Studies from this laboratory (1–3) and others (4, 5) have indicated that testicular extracts are capable of oxidizing progesterone to testosterone and acetic acid. Preliminary data from this laboratory indicated that the enzymatic activity was associated with the microsomes, that reduced triphosphopyridine nucleotide (TPNH[1]) and oxygen were essential for the reactions, and, further, that cyanide was impressively stimulatory. This report deals with some properties of the four enzymes thus far implicated; namely, (1) progesterone → 17α-hydroxyprogesterone; (2) 17α-hydroxyprogesterone → androstenedione + acetic acid; (3) androstenedione ⇌ testosterone; and (4) 17α-hydroxyprogesterone ⇌ Δ^4-pregnene-17α,20β-diol-3-one (and some of their properties).

EXPERIMENTAL

Substrates—Progesterone-4-C^{14} and progesterone-21-C^{14} were obtained from commercial sources and were found to be chromatographically pure. Δ^4-Pregnene-17α,20β-diol-3-one, as well as the 20α isomer, was obtained from Dr. T. Gallagher and Dr. I. Salamon of the Sloan-Kettering Institute for Cancer Research. All other steroids were obtained from commercial sources. Melting points and chromatographic purity were checked for each of the substrates.

All of these water-insoluble substrates were dissolved in ethanol, and small volumes of the solutions were added directly to the incubation mixtures. After incubation, proteins were precipitated by 2 volumes of ethanol, and the products were isolated from the filtrate.

Isolation and Identification Procedures—In experiments in which progesterone-21-C^{14} was used, radioactive acetic acid was isolated directly from the incubation mixture by distillation. The acetic acid was further separated by two paper chromatographic systems, either as the free acid (6)

* Supported by the United States Public Health Service, grant No. RG4758.

† Markle Scholar in Medical Sciences.

[1] The following abbreviations are used in this paper: TPNH, reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane; DPNH, reduced diphosphopyridine nucleotide; TPN, oxidized triphosphopyridine nucleotide; PP, sodium pyrophosphate.

or as the ammonium salt (7). Final identification was accomplished by the addition of carrier acid and the preparation of two derivatives, the anilide and the *p*-toluide. These derivatives were recrystallized to constant specific activity.

To distinguish further between formic and acetic acids, a Duclaux constant was obtained (Table I). Slaunwhite and Samuels (4) reported that they found formic acid in a similar testicular extract; however, no formic acid was obtained in our incubations.

Radioactive Steroid Products—In experiments in which progesterone-4-C^{14} was the substrate, the steroids were extracted with ether and then separated by the paper chromatographic procedures of Zaffaroni and Burton (8) and Bush (9). After separation by these two different methods, the four enzymatic products, 17α-hydroxyprogesterone, testosterone, androstenedione, and Δ^4-pregnene-17α,20β-diol-3-one, were eluted from the chromatograms, carrier steroids were added, and crystallizations to constant specific activity were carried out (Table I). Δ^4-Pregnene-17α,20β-diol-3-one is easily separable from its 20α isomer in the chromatographic system of Zaffaroni (Table II). The dihydroxyketopregnene was further identified by the isolation of radioactive acetaldehyde as the product of the bismuthate oxidation of the side chain of the radioactive dihydroxyketopregnene. The acetaldehyde was trapped as its 2,4-dinitrophenylhydrazone, and this product separated on the paper chromatographic system previously described (10). Carrier hydrazone was then added, and this was crystallized to constant specific activity. (The only two steroids of this group which are difficult to separate are testosterone and 17α-hydroxyprogesterone; however, these two steroids were separable in a modified Zaffaroni paper chromatographic system; *i.e.*, the paper strips were dipped in a mixture of propylene glycol and methanol, ratio 1:3 rather than 1:1) (Table I). In experiments with non-labeled substrates, the steroids were determined after separation and elution by measuring their absorption at 240 mμ. Since these testicular extracts contain no 3-keto reductases, quantitative recoveries could be obtained with the spectral assay. The 17-ketosteroids were also determined colorimetrically by the Zimmermann reaction (11).

Appropriate paper chromatographic blanks without substrate were run in all cases. To insure further that the 240 mμ absorption represented only steroid absorption, the spectra of ethanolic eluates between 210 and 300 mμ were obtained by a model 11 Cary recording spectrophotometer. In all experiments, the ultraviolet absorption in this region was characteristic of the Δ4,3-keto structure of the steroids.

Enzyme Preparation

Preparation of Microsomes—All four enzymes were found to be much more active in their particulate state. These particles were obtained by

homogenizing (Potter-Elvehjem homogenizer) the decapsulated testes of guinea pigs in 0.02 M Tris-HCl buffer (pH 7.0). The large inactive parti-

TABLE I

Radioactivity of Products and Derivatives Obtained from Radioactive Progesterone

Compound		No. of crystallizations	Solvent	Radioactivity*		
				Weight	Total counts	
	m.p.†			mg.		c.p.m. per mg.
Androstenedione	172	1	Methanol	33	2000	60
	174	2		16	930	58
	174	3		14.1	798	57
	173	4		10.2	552	55
17α-Hydroxyprogesterone	221	1	Methanol	45	5000	111
	223	2		25	2560	102
	223	3		9.7	1052	107
Δ⁴-Pregnene-17α,20β-diol-3-one	195–202	1	Methanol-water	10.1	3500	347
	195–200	2		5	1640	324
	196–200	3		1.7	560	330
Acetaldehyde-2,4-dinitrophenylhydrazone	165	1	Ethanol	27	600	22
	165	2		17	345	20
	167	3		6.1	115	19
Testosterone	154	1	Methanol	42	4500	107
	155	2		21.2	2050	96
	155	3		9.7	1025	105
Anilide of acetic acid	112	1	Methanol-water	400	6000	15
	112	2		148	2010	13
	114	3		22	340	15
		4		6.1	101	16
p-Toluide of acetic acid	145	1	Methanol-water	200	4000	20
	147	2		95	1760	18.5
	147	3		26	460	17.6

Duclaux constant of radioactive acetic acid (3800 counts)

1st 10 ml.	Counts, 140	Found,‡ 5.6	Theory,‡ 6.8
2nd 10 "	" 166	" 6.6	" 7.1
3rd 10 "	" 177	" 7.6	" 7.4

* Counts per minute per mg. represent the counts corrected to infinite thinness.

† All melting points were performed on a Fisher-Johns electric apparatus.

‡ Ratio.

cles were removed from this homogenate by centrifugation at 10,000 × *g* for 15 minutes, and the small active particles were obtained from this supernatant fluid by centrifugation at 100,000 × *g* for 20 minutes. The latter particles were then suspended by gentle homogenization in sufficient

TABLE II

Localization of Testicular Enzymes

Enzyme	Radioactive products recovered	
	17α-Hydroxy-progesterone	Acetate
	per cent	*per cent*
Supernatant fluid, 100,000 × *g*	7	0
Small particles, 10,000–100,000 × *g*	20	25
Large " 2000–10,000 × *g*	0	0
Supernatant fluid		
0–50% $(NH_4)_2SO_4$ fraction*	15	6
Soluble 0–50% $(NH_4)_2SO_4$ fraction	0	0
Insoluble 0–50% $(NH_4)_2SO_4$ fraction	15	14
Small particles + supernatant fluid	15	70
Washed small particles − supernatant fluid	2	0
" " " + " "	20	60

Enzyme, the amount of enzyme used per incubation corresponded to that amount of material obtained from one-half of a guinea pig testicle. Substrate, 0.3 μmole of progesterone-4-C^{14} (10,000 counts). Incubation, 0.02 M Tris-HCl, pH 7.0, at 30° for 1 hour. Gas phase, air. Additions, TPN 0.02 μmole, glucose-6-phosphate dehydrogenase 0.5 unit (see the footnote below). Glucose 6-phosphate 5.0 μmoles, and NaCN 10^{-5} M. Washed small particles; particles washed twice with 10 ml. of 1 M NaCl.

* The supernatant fluid was precipitated with $(NH_4)_2SO_4$ (50 per cent saturated), and this precipitate, resuspended in Tris buffer and then recentrifuged at 100,000 × *g* for 15 minutes (see "Insoluble 0–50 per cent $(NH_4)_2SO_4$ fraction"), contained the enzymatic activity. The TPNH-generating system was equally active in all fractions.

Tris buffer to give an emulsion with an optical density of 1.0 at 280 mμ. The constituents of these microsomes were previously reported (12).

Fractionation Procedures—Attempts by various procedures to dissolve these microsomes failed, but it was observed that the inactive soluble 100,000 × *g* supernatant fluid could be made active by precipitating the proteins either with salt or ethanol or by dialysis against buffer. However, in each instance it was found that the generated activity resided in the insoluble precipitates produced by these procedures (Table II). These observations appeared to indicate that, although these enzymes were pres-

ent in a soluble form, they were active only in the presence of particles. It was further observed that these active particles could be inactivated by washing them repeatedly with either 0.02 M Tris buffer or with 1 M NaCl and that their enzymatic activity could be regenerated by reexposure to more 100,000 × *g* supernatant fluid. From this observation it appeared possible to fractionate the enzymes soluble in the supernatant fluid and to assay them in the presence of the washed inactive particles. This proved to be the case. However, since these enzymes were usually largely inactivated by salt, ethanol, or isoelectric precipitations, the most useful method of separation thus far found was adsorption and gradient elution, with the hydroxyapatite columns of Tiselius *et al.* (13). Columns, 4 × 1 cm., containing 75 gm. of hydroxyapatite, were sufficient to adsorb and to separate over 90 per cent of the protein obtained in the supernatant fluid from six guinea pig testicles (about 20 ml. in volume). Elution and separation were effected by eluting these columns with four 10 ml. fractions of phosphate buffer, pH 6.8, of increasing ionic strength; Fraction 1, 0.001 M phosphate buffer; Fraction 2, 0.01 M; Fraction 3, 0.1 M; and Fraction 4, 1.0 M. The 1 M buffer removed most of the remaining protein, including hemoglobin (Table III). The last two fractions were then dialyzed against sufficient water to make the final phosphate concentration 0.05 M. As shown in Table III, the two reductases as well as the two oxidases could be easily separated; however, the androstenedione reductase contaminated both oxidase fractions. Since these fractions are still unstable (50 per cent of activity lost in 24 hours), no further purification has been obtained. Although the two reductases were initially present in the supernatant fluid (100,000 × *g*), the ratio of their activities could be greatly accelerated by the addition of the washed microsomes.

Incubations—These were routinely made in closed vessels at 30° with shaking at pH 7.0 in either 0.01 M phosphate or 0.02 M Tris buffer. Consumption of oxygen was measured by the conventional Warburg technique.

Results

Guinea pig and rat testicles were used as the source of enzymes. Rat testicles were appreciably active only after injection of the animals daily for 2 weeks with 25 units of chorionic gonadotropin (Upjohn) per day. Guinea pig glands were equally active both before and after injections of chorionic gonadotropin. Since the rat glands contained only slight amounts of androstenedione reductase, the cleavage product from progesterone or 17α-hydroxyprogesterone was primarily androstenedione, whereas in the guinea pig testosterone was the cleavage product isolated. However, the androstenedione reductase obtained from the guinea pig could be

inhibited at pH 8.5, and, under these conditions (the cleavage enzyme is still appreciably active at pH 8.5), the product obtained with the guinea pig preparation was also androstenedione (Table VIII). Table IV indi-

TABLE III

Enzyme Fractionation

Enzyme fraction	Protein	Enzymes present	Activity
	mg. per ml.		*per cent*
Microsomes + supernatant fluid	1.2	All 4	100
Microsomes		" 4	60
Washed microsomes*		Traces	>1
" " + supernatant fluid	1.2	All 4	86
Supernatant fluid only	1.2		>1
Washed microsomes + Fraction 0	0.04	Traces	>1
Washed microsomes + Fraction 1	0.02	20β-Reductase	70
Washed microsomes + Fraction 2	0.04	"	15
Washed microsomes + Fraction 3	0.22	Androstenedione reductase,	60
		17α-hydroxylase	90
Washed microsomes + Fraction 4	0.66	Androstenedione reductase,	30
		17α,20-keto cleavage enzyme	50

Enzyme, all incubations contain that portion of protein which was obtained from one-half of a guinea pig testicle by the fractionation procedures. The fractions represent eluates from hydroxyapatite columns (see the text). Incubations, Tris-HCl buffer, pH 7, for 30 minutes at 30° with air as the gas phase. Additions, TPN 0.02 μmole, glucose 6-phosphate 5.0 μmoles, glucose-6-phosphate dehydrogenase 0.5 unit. Substrate, 0.3 μmole of each substrate for each enzyme was added in 0.02 ml. of ethanol, and the products were assayed, after chromatographic separation, by their adsorption at 240 mμ. Per cent activity, amount of product produced in 30 minutes expressed as per cent of the activity of microsomes plus the supernatant fluid. Assays were for the 20β-reductase, 17α-hydroxylase, 17,20-cleavage enzyme and androstenedione reductase. To assay the 20β-reductase, it was necessary to inhibit the 17,20 cleavage enzyme. This could be done under anaerobic conditions.

* Microsomes washed twice with 10 ml. of NaCl (1 M).

cates that TPNH and O_2 are specific and are the only cofactors necessary for the oxidases, whereas TPNH is the only cofactor necessary for the reductases. Cyanide, 10^{-4} M, pyrophosphate, 0.002 M, and carbon monoxide, 5 per cent, had stimulating effects on all of the enzymes. These effects were probably the result of the inhibition of cytochrome oxidase, which

was present in these particles (as is TPNH cytochrome *c* reductase) (11). Although these particles contain only trace amounts of porphyrins (12), cytochrome oxidase, as measured by the oxidation of reduced cytochrome *c*,

TABLE IV

Cofactor Requirements

Substrate additions	Progesterone-21-C^{14}			17α-Hydroxyprogesterone		Androstenedione
	17α-Hydroxyprogesterone	Acetate	3-Ketopregnenediol*	Testosterone	3-Ketopregnenediol*	Testosterone
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
O	4	0	0	0	0	2
TPN 1.0 μmole	10	0	5	0	7	15
TPNH 1.0 μmole	15	35	5	30	10	80
DPNH 1.0 "	4	0	7	0	3	11
Peroxide generators,† 3 μmoles	4	0	0	0	0	6
TPNH, 1 μmole + N_2	10	4	25	4	29	75
" 1 " + CN 10^{-5} M	4	90	5	80	7	100
" 1 " + PP, 2 μmoles	8	85	0	80	10	100
" 1 " + CO 5 per cent	15	70	10	50	15	100
TPNH, 1 μmole + malate, 5 μmoles	20	75	Trace	50	17	100
TPNH, 1 μmole + NaCl, 0.02 M	15	60	10	55	20	100
" 1 μmole + N_2 + NaCl, 0.02 M	20	0	45	5	50	100
TPNH, 1 μmole + catalase, 0.5 mg	21	37	7			

Enzyme, microsomes obtained from one-half of a guinea pig testicle per incubation. Substrates, 0.3 μmole of each, added in 0.02 ml. of ethanol. Incubation, 1 hour at 30° in 0.02 M Tris-HCl at pH 7 in a final volume of 2 ml. Products, all steroids assayed after chromatographic separation by their absorption at 240 mμ. The radioactive acetate was counted. The numbers refer to the per cent of the added substrate.

* Δ^4-Pregnene-17α,20β-diol-3-one.

† The peroxide generators used were glucose oxidase and xanthine oxidase (hypoxanthine used as a substrate for the xanthine oxidase).

is present in these particles, and also is inhibited by 10^{-5} M NaCN. These particles are also much more active in the presence of salt (Na_2SO_4, K_2SO_4, $NaHPO_4$, KCl, NaCl, $MgCl_2$) in concentrations of 0.002 to 0.02 M. Table V shows that all four enzymes are more active in the presence of salt.

Stoichiometry—Table VI shows that each oxidative step, *i.e.* the hydroxylase and the cleavage enzyme, uses approximately 1 mole of O_2 per

1 mole of TPNH. The results are complicated by the fact that the enzymes could not be separated adequately, by the presence of non-specific TPNH oxidases in these microsomes, and by the presence of the 17α-reductase which is also competing for the TPNH. However, the amount of TPNH used by the 17α-reductase, *i.e.* the amount of testosterone formed, is known, and, by subtracting this amount from the total, the amount of TPNH used in the oxidative steps can be obtained. The data indicate that the oxidation of 1 mole of progesterone to androstenedione

TABLE V

Stimulation of Microsomal Enzymes by Salts

		Substrate: 17α-hydroxy-progesterone	Substrate: progesterone-21-C^{14}
		Testosterone formed	Acetate formed
	M	*per cent*	*per cent*
Control		14	10
Na_2SO_4	0.0005	16	10
"	0.002	92	70
"	0.02	85	50
$NaHPO_4$	0.002	90	60
"	0.02	60	15
PP	0.002	95	100
"	0.02	25	20
KCl	0.02	60	30
NaCl	0.02	60	30
K_2SO_4	0.02	90	40
$MgSO_4$	0.02	40	30
"	0.002	82	35

Each incubation contained 0.3 μmole of substrate, 0.8 μmole of TPNH and NaCN, 10^{-5} M in 2 ml. volume. The enzymes were microsomes obtained from one-half of a guinea pig testicle per incubation in Tris-HCl buffer, pH 7. Gas phase, air, at 30°, for 1 hour. The products were assayed in the same manner as in Table IV.

requires approximately 2 moles each of O_2 and TPNH, and that the oxidation of 17α-hydroxyprogesterone to androstenedione requires 1 mole of each (3). Confirmation of this 1:1 ratio of TPNH and O_2 was also obtained by using washed microsomes plus the supernatant fractions of the hydroxyapatite columns. Since the stoichiometry implied that these enzymes were peroxidatic, other sources of enzymatic peroxide, xanthine oxidase and glucose oxidase, were used; however, these peroxide generators (Table IV) could not replace the need for TPNH. Moreover, catalase did not inhibit these oxidative enzymes. Therefore, if peroxide is the oxidant, it is not free peroxide, but peroxide generated in or on these particles by TPNH.

Inhibitors—Table VII indicates that all of these enzymes, in the presence of microsomes, are inhibited by *p*-chloromercuribenzoate but not by iodoacetate. Interpretation of these data on inhibitors is difficult because of the fact that many of these inhibitors tended either to clump or to precipitate the microsomal emulsion; thus the results of such data may be interpreted simply by means of their "detergent" activity on the particles.

TABLE VI

Stoichiometry of Enzymatic Reactions

	O_2 used	TPNH used	Substrate oxidized	Substrate reduced	Product	TPNH used for oxidation/ O_2 used
	μmole	*μmoles*	*μmole*	*μmole*	*μmole*	
Control (no substrate)..	0.11	0.15			0	1.3
" (" TPNH)....	0.08					
Progesterone, 1 μmole...	0.76	1.0	0.55	0.2	17α-Hydroxyprogesterone	
					Testosterone, 0.2	0.98
					3-Ketopregnenediol,* 0.05	
17α-Hydroxyprogesterone, 1 μmole.....	0.51	1.1	0.46	0.4	Testosterone, 0.4	1.14
Androstenedione, 1 μmole.....	0.18	1.1		0.95	" 0.91	
Testosterone, 1 μmole...	0.03	0.08	0.05		Androstenedione, 0.05	

Enzyme, microsomes from 1 guinea pig testicle. Each flask contained 1 μmole of substrate (added in 0.02 ml. of ethanol), TPNH, 1.3 μmoles, NaCN, 10^{-4} M, Tris buffer 0.02 M, pH 7, to a final volume of 2 ml. at 30°. TPNH was measured by titration of the incubation mixture with 2,6-dichlorophenol-indophenol at the end of the incubations (3). O_2 was measured with standard Warburg flasks and substrates were measured, after separation by paper chromatography, by their adsorption at 240 mμ.

*Δ^4-Pregnene-17α,20β-diol-3-one.

However, since inhibitory phenomena are not present for all enzymes in the microsomes, the effects may be specific. The hydroxylase and the reductases are stable to most of the inhibitors, but the cleavage enzyme is inhibited by a number of reagents like Versene, and other metal-chelating agents such as diethyl dithiocarbamate, α,α-dipyridyl, and *o*-phenanthroline, as well as heavy metals such as iron and copper. Although other hydroxylases are known to be metal-containing, for example phenol oxidase (14), these data seem to imply that the cleavage enzyme rather than the steroid hydroxylase is a metal-containing enzyme.

Effects of pH—Table VIII indicates the apparent pH optimum of the various enzymes in microsomes. The cleavage enzyme is most active at pH 7, whereas the hydroxylase is still quite active at pH 8.5. The 20β reductase and androstenedione reductase are inhibited at alkaline pH values and are active at values as low as pH 6.

Effects of Hydrolytic Enzymes—To ascertain the type of structure or membrane which maintained the insolubility and catalytic activity of these

TABLE VII

Inhibitors of Steroid Enzymes

Inhibitor	Per cent product formed in 30 minutes		
	17α-Hydroxylase	Cleavage enzyme	Androstenedione reductase
Control	40	50	80
p-Chloromercuribenzoate, 10^{-3} M	0	0	10
Versene, 10^{-3} M	35	5	80
Diethyl dithiocarbamate, 10^{-3} M	60	0	80
Cyanide, 10^{-4} M	65	80	100
Pyrophosphate, 10^{-3} M	60	75	100
α,α-Dipyridyl, 10^{-3} M	45	0	80
o-Phenanthroline, 10^{-3} M	45	25	80
Iodoacetate, 10^{-3} M	0	50	70
Nitrogen	0	0	100
Fe^{++}, 2×10^{-4} M	40	0	80
Cu^{++}, 2×10^{-4} M	40	0	0
Catalase, 0.5 mg	35	43	85

Enzyme, washed microsomes obtained from one-half of guinea pig testicle per flask, plus the appropriate purified enzyme from hydroxyapatite columns (see the text). Incubation was performed at pH 7 (Tris-HCl) in 2 ml. of final volume at 30° in an open flask. Each flask contained 0.3 μmole of the various substrates, 0.6 μmole of TPNH, and NaCN 10^{-4} M. The products were assayed after chromatographic separation by their absorption at 240 mμ.

particles, preliminary treatment of these microsomes with trypsin, ribonuclease, and pancreatic lipase was performed. Particles obtained from guinea pig testicles were incubated at 30° (pH 7, Tris buffer) with 1 mg. of each of the above enzymes and then again sedimented. The amount dissolved was assayed by measuring the increase of absorption at 260 and 280 mμ in the supernatant fluid. Enzyme activity was assayed in the usual way in the resuspended particles. Of the three hydrolytic enzymes used, only lipase caused any leaching of ultraviolet-absorbing material, and it was, likewise, the only enzyme which inactivated the steroid enzymes. If, however, particles which had been treated with lipase were

then treated with trypsin and ribonuclease, a further marked increase in ultraviolet-absorbing material was obtained in the supernatant fluid (Table IX). This implies that the surface of these particles is largely fat and that this fat is necessary for enzymatic activity, and furthermore, that this fatty surface protects these particles from the hydrolytic activity of trypsin and ribonuclease.

Sequence of Enzymatic Events—With these impure enzymes, two questions arose: was it the Δ^4-pregnene-17α,20β-diol-3-one or the 17α-hydroxyprogesterone which was the substrate that was cleaved; and was acetate

TABLE VIII

Effects of pH on Steroid Enzymes

pH	Substrate: Progesterone-21-C^{14}, 0.2 μmole			
	Products formed		3-Ketopregnene-diol*	Androstenedione
	Acetate	17α-Hydroxy-progesterone		
	per cent	*per cent*	*per cent*	*per cent*
6.0	4	12	20	0
6.5	20	12	25	0
7.0	35	18	15	0
7.5	15	22	11	5
8.5	11	33	0	12†

Enzyme, microsomes were obtained from one-half of a guinea pig testicle. Incubation was performed for 30 minutes at 30° in Tris-HCl, pH 7, volume 2 ml. Gas phase, air. Products were assayed in the same manner as in Table IV. Additions, 0.6 μmole of TPNH.

* Δ^4-Pregnene-17α,20β-diol-3-one.

† Because of the difficulty in separating progesterone from androstenedione, the formation of androstenedione was noted, 17α-hydroxyprogesterone being used as the substrate, and the same results were obtained.

the actual cleavage product? The first question was answered when the 3-ketopregnenediol became available in quantity. The equilibrium of the 20β reductase in the presence of excess TPNH was in favor (90 per cent) of the reduced product, and it could be shown under these conditions that very little cleavage could be effected, as compared to a similar incubation with 17α-hydroxyprogesterone as substrate (Table X). Moreover, in incubations performed at pH 8.5, cleavage of 17α-hydroxyprogesterone could be obtained, but under these conditions microsomes could neither reduce 17α-hydroxyprogesterone to Δ^4-pregnene-17α,20β-diol-3-one nor cleave the side chain of the latter compound or its 20α epimer. These observations made it likely that the actual substrate undergoing cleavage is 17α-hy-

TABLE IX

Effect of Lipase, Ribonuclease, and Trypsin

Substrate: 0.2 μmole of progesterone-21-C^{14}

	Products formed			
	17α-Hydroxy-progesterone	3-Keto-pregnenediol*	Acetate	Material released‡ 260 mμ
	per cent	*per cent*	*per cent*	
Control	10	0	80	
Ribonuclease-treated,† 1 mg.	10	20	55	0.05
Trypsin-treated,† 1 mg.	12	15	59	0.03
Lipase-treated,† 1 mg.	30	31	5	0.350
Lipase + ribonuclease + trypsin	5	10	0	0.95

Enzyme, microsomes obtained from one-half of a guinea pig testicle, preincubated with 1 mg. of each of the hydrolytic enzymes for 30 minutes at 30°, pH 7, and then resedimented at 100,000 × *g* for 20 minutes. The increase over controls in adsorption at 260 mμ was obtained on each supernatant fluid. Additions, 0.5 μmole of TPNH. Incubations, pH 7 (Tris 0.02 M), for 30 minutes at 30° in open flasks. Products, assayed in same manner as in Table IV.

* Δ^4-Pregnene-17α,20β-diol-3-one.

† The hydrolytic enzymes were all commercially obtained and were enzymatically active.

‡ The material released had the typical broad adsorption peak at 260 mμ of nucleotides.

TABLE X

Substrate for Cleavage Enzyme

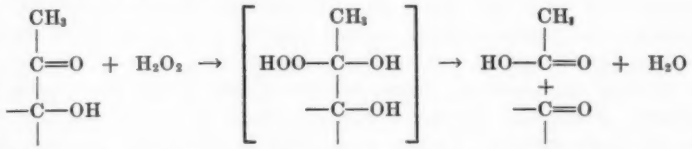
Substrate	Cleavage*	17α-Hydroxy-progesterone	Δ^4-Pregnene-17α,20β-diol-3-one
	per cent	*per cent*	*per cent*
17α-Hydroxyprogesterone, pH 7	66		15
" " 8.5	22		0
Δ^4-Pregnene-17α,20β,diol-3-one, pH 7	10	11	
" " 8.5	0	0	
Δ^4-Pregnene-17α,20α,diol-3-one, pH 7	0	0	0

Enzyme, microsomes obtained from one-half of a guinea pig testicle. Incubation, in same manner as in Table IV. Addition, TPN 0.2 μmole, glucose 6-phosphate 5 μmoles, glucose-6-phosphate dehydrogenase 0.2 unit, NaCN 10^{-5} M. Products, assayed in same manner as in Table IV. All substrates were added in 0.2 μmole amounts.

* The cleavage product was testosterone at pH 7, but 90 per cent androstenedione at pH 8.5.

droxyprogesterone. Also, the fact that the 17-hydroxyl function is α in the substrate and β in the product strongly implies that the 17-ketone had to be the intermediate.

The answer to the second question is less clear. In repeated experiments, with progesterone-21-C^{14} and the partially purified enzymes, the only radioactive non-steroidal material present at the end of the incubation was acetic acid. Many attempts to isolate radioactive acetaldehyde or ethanol, by use of trapping agents or carrier acetaldehyde, have failed. Also, the stoichiometric measurements, which indicate that the oxidation equivalent of 1 mole of H_2O_2 is needed per mole of substrate cleaved, imply a peroxidatic type oxidation, thus:



If the above mechanism is correct, both fragments, certainly the acetic acid and possibly the androstenedione, should contain oxygen derived from the atmosphere. O^{18} measurements to test this hypothesis have been made, but, because of the low activity of the enzymes and insufficient amounts of the products, the O^{18} measurements are still equivocal.

DISCUSSION

These data indicate that there are at least four enzymes, androstenedione reductase, 20-keto reductase, 17α-hydroxy-20-keto cleavage enzyme, and 17α-hydroxylase, involved in the production of testosterone from progesterone in testes. All these require TPNH (DPNH was inactive) and two require molecular oxygen. These two which require oxygen, 17α-hydroxylase and the 17α-hydroxy-20-keto cleavage enzyme, are found in microsomes and were appreciably active only in the presence of particles. However, the two reductases, androstenedione reductase and the 17α-hydroxy-20-keto reductase, are active in the soluble state, but their activity was impressively stimulated by the presence of washed inactive particles. It has also been demonstrated that these particles are non-specific in that they can be generated from the soluble supernatant fluid by such procedures as dialysis and salt precipitation. It has been shown previously that these particles have a great affinity for water-insoluble substances (12) such as steroids and Amphenone B, and can quantitatively remove these substances from aqueous solution. Therefore, the ability of these particles to stimulate these steroid enzymes could be due to their surface activ-

ity; *i.e.*, their ability to adsorb the steroids as a monomolecular film, thus effecting emulsification of the globules of steroids which were added as substrate. By using these particles as emulsifying agents, partial purification of the four enzymes was accomplished by chromatography on columns of hydroxyapatite. Many other procedures for fractionating proteins proved to be ineffective.

Since two of the enzymes involved, the hydroxylase and the cleavage enzyme, require molecular oxygen, an understanding of the mechanism of this type of oxidation was sought. The TPNH-O_2 stoichiometry of each enzyme indicates that the equivalent oxidative power of hydrogen peroxide is necessary. A great number of other similar enzymes have now been described and have been shown to add molecular oxygen to various substrates such as phenol oxidases, steroid oxidases (15), tryptophan oxidase (16), and lipoxidase (17), but no clear understanding of the mechanism of any of these has evolved. Some are stimulated by catalase; some are inhibited. Some can use any source of enzyme-generated peroxide and some are quite specific. Copper is involved in the phenol oxidases, iron in tryptophan oxidase, but neither metals nor sulfhydryl groups are involved in the enzymatic action of lipoxidase. The studies reported here on two steroid oxidases indicate that two different types of enzymatic mechanisms are present, although both require molecular oxygen as well as a specific source of reductive power, *i.e.* TPNH. Both enzymes, by stoichiometric measurements, require either hydroxyl radicals or hydrogen peroxide, but they most likely require the free radicals since neither catalase nor enzymatically generated peroxide has any effect. However, it was previously shown that these particulate preparations contain large quantities of catalase activity (12), and the addition of more catalase would be expected to be without effect (Table IV). Metals do not seem to be involved in the action of the 17α-hydroxylase because metal-binding agents are without effect, and it is not likely that porphyrins are involved, for there is no measurable (photometrically) amount of porphyrin present (12). These particles do, however, contain sizable quantities of uncharacterized flavins (12). Perhaps these flavins are the coenzymes involved in the reduction of the oxygen; however, addition of flavin mononucleotide, flavin adenine dinucleotide, or riboflavin was without effect on either enzyme.

The cleavage enzyme differs from the hydroxylase in that it is inhibited by metal-binding agents, *i.e.* Versene, diethyl dithiocarbamate, and α,α-dipyridyl, but not by cyanide or pyrophosphate. The fact that both enzymes are not inhibited but actually stimulated by cyanide as well as pyrophosphate makes it uncertain that metals are involved. All these interpretations are, of course, open to question because of the particulate nature of these enzyme preparations.

Finally, the fact that these steroid as well as other enzymes (previously demonstrated) (12) can be activated by surface-active agents such as intracellular particles, and the fact that agents which are adsorbed by these particles, such as Amphenone B, digitonin, and other detergents (3), can also affect the rate of enzymatic activity, plus the fact that the ionic strength of the medium exerts an appreciable effect on these phenomena, make very attractive the idea that these microsomal particles may exert considerable control over intracellular phenomena. Similar surface phenomena at cell membranes must also undoubtedly occur. Such phenomena have been hypothesized for the action of the fat-soluble vitamin D on the gut and bone (18). Data reported here seem both to indicate that the cell membrane is not the only surface of the cell which has regulatory functions and to imply that surface phenomena within the cell are equally important. Fraser and Kaplan (19) from their studies on the activation of catalase in yeast, and Robert and Polonovski (20) from their work on the activation of the lipoprotein particles of xanthine oxidase in milk, have drawn similar conclusions.

SUMMARY

Four enzymes from testes concerned with the oxidation of progesterone to testosterone and acetic acid have been partially purified. All of them require reduced triphosphopyridine nucleotide and two require oxygen. Each is markedly catalyzed by the presence of cytoplasmic particles. Δ^4-pregnene-17α,20β-diol-3-one has been identified as one of the enzymatic products.

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A STUDY OF A MUCOLIPIDE FROM OX BRAIN*

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(Received for publication, January 13, 1958)

We should like to subsume under the group designation "mucolipide" the complex lipide polymers that combine constituents commonly considered characteristic of the cerebrosides with others usually assigned to the mucoids. They are soluble in water, but also in organic solvents, and they contain fatty acid, a sphingosine-like base, a hexose, also amino sugar, sometimes amino acids, and, most significantly, sialic acid or a related substance. This has been pointed out recently in notes dealing briefly with the subject of this communication (1, 2).[1]

Lipides of the general type under discussion here, *i.e.* compounds related to the cerebrosides, but soluble in water and yielding a violet color with Bial's reagent or a direct color reaction with *p*-dimethylaminobenzaldehyde without previous treatment with alkali, have been encountered not infrequently in the older literature. Since what appears to be their first description, by Landsteiner and Levene (4, 5), similar substances were found in spleen (6), normal brain (7), and in the brain of cases of Niemann-Pick disease (8) and of Tay-Sachs disease (9). Two representatives of the mucolipide group have been investigated in some detail. (1) The "gangliosides," a name first given to mucolipides isolated from brain tissue of instances of lipidoses (10) and later extended, perhaps without justification, to material from normal nervous and other tissues; and (2) "strandin," a high molecular lipide complex from ox brain (11). Folch and his collaborators (11) deserve great credit for pointing out the existence in nervous tissue of a lipide derivative of high molecular weight. For the isolation of this material, they employed a much milder procedure than is customary

* This work has been supported by research grants from the National Institutes of Health, United States Public Health Service, the Life Insurance Medical Research Fund, and the Rockefeller Foundation.

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[1] It should be noted that the term "mucolipide" is used here in a much more discriminative sense than was done in a review (3) in which it served to classify a large number of chemically unrelated lipopolysaccharides, mostly of bacterial origin and of unknown composition.

in lipide chemistry, and it could have been conceivable that the method of its isolation had to do with the original claim (11) that strandin was virtually devoid of the chromogen reacting in the orcinol and Ehrlich tests and apparently characteristic for mucolipides. This claim has, however, been shown to be unfounded (1, 2, 12) and was later retracted (13). Other substances chemically, and perhaps even physiologically, related to the mucolipides are "globoside" from the stroma of human red cells, a substance which, however, lacks the chromogen (14), and "hematoside" from horse red cell stroma, which contains no amino sugar (15).

The present investigation was prompted by several observations. It was found that a lipide preparation obtained by the extensive purification of a crude fraction isolated from the gray matter of brain by the "partition dialysis" method (11) contained a considerable proportion of the chromogen mentioned before (1). This preparation was, moreover, found to differ from ganglioside preparations in showing a marked inhibitory action on the agglutination of chicken erythrocytes by influenza virus (1). What appeared of even more general biological interest was the consideration that the high molecular weight and complex composition of the mucolipides make it attractive to regard them as potential carriers of species specificity, owing to the multiple possibilities of differences in the sequential arrangement of their constituents similar, in that respect, to the nucleic acids and proteins (2). The present study is concerned with the properties of an ampholytic mucolipide isolated in homogeneous form from ox brain. Mucolipides from the brain tissues of other species will, it is hoped, form the subject of future reports.

EXPERIMENTAL

Preparation of Mucolipide

Isolation of Crude Material—The crude mucolipide was isolated from ox brain by a modification of the partition dialysis procedure (11). Fresh "gray matter" was treated for 2 minutes in a Waring blendor with a 10-fold amount (v/w) of chloroform-methanol (2:1, v/v) previously cooled to 0°. The resulting mixture was immediately filtered by suction and stored in the cold. The filtrate was subjected to dialysis through cellophane in the cold for 4 days against eight changes of a 30-fold volume of distilled water. The clear supernatant aqueous phase of the dialysis residues was collected and rapidly concentrated to approximately 1 per cent of its volume in a flash evaporator at a bath temperature of 30°. The concentrate was then again dialyzed in the cold against a 100-fold volume of distilled water under the conditions described above. An insoluble deposit was removed by centrifugation at 2100 × *g* for 30 minutes. In five separate preparations, the lyophilized supernatant liquid yielded an average weight of

crude mucolipide equal to 0.34 per cent of the wet weight of tissue extracted. Preparations at this stage of purity will be designated as "Stage I."

Inhomogeneity of Crude Mucolipide—Strandin has been reported (11) to migrate in the Tiselius apparatus as a single negatively charged entity at pH values ranging from 8.6 to as low as 1.38; ultracentrifuge and x-ray diffraction (16) studies, however, gave evidence of inhomogeneity. We have found that the crude mucolipide preparation at the strandin stage can be separated at pH 3.7 into a number of negatively charged components and a mucolipide fraction acquiring a positive charge. The separation was effected by electrophoresis (Reco model E-800-2) on filter paper (Schleicher and Schuell No. 589) for 1.5 hours, with the use of a supporting electrolyte of 0.05 M formate (pH 3.7) and of a gradient of 13.3 volts per cm. The results are shown graphically in Fig. 1.

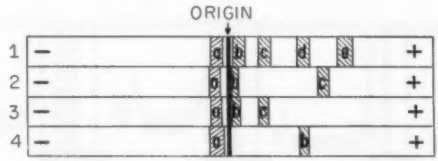

FIG. 1. Paper electrophoresis of crude mucolipide in 0.05 M formate at pH 3.7. The diagram represents four numbered lanes treated as follows after the completion of the run: (1) sprayed with $AgNO_3$ and NH_4OH and then irradiated with ultraviolet light (17); (2) viewed under ultraviolet light; (3) developed with ninhydrin; (4) developed with an orcinol-trichloroacetic acid reagent (18). The mucolipide is represented by band "a" on each strip.

Purification—First, the crude mucolipide (Stage I) was again subjected to partition dialysis, as the relatively large amounts of various lipides present in the original tissue extract could have carried along water-soluble contaminants. A 3 per cent solution of crude mucolipide in chloroform-methanol (2:1, v/v) was permitted to dialyze for 48 hours in the cold against 50 volumes of distilled water and, for 5 hours more, against 200 volumes. The aqueous phase yielded approximately one-half of the weight of the original crude mucolipide. Mucolipide preparations that had been repartitioned once or more will be referred to as "Stage II." Paper electrophoresis now showed a mucolipide band and one slowly moving negatively charged band which reacted with ninhydrin. Salt dissociation was employed for the removal of this last remaining contaminant. A 5 per cent (w/v) solution of repartitioned mucolipide in chloroform-methanol (2:1, v/v) containing 10 per cent (w/v) $CaCl_2 \cdot 2H_2O$ was filtered through Whatman No. 44 paper and subjected to partition dialysis as before. The aqueous phase, when taken to dryness in the frozen state in a vacuum,

yielded a glistening, very pale yellow, laminated mass, again accounting for approximately one-half of the starting material. It corresponds to "Stage III" of purity. This substance, which served in the following physical studies, showed a single band upon paper electrophoresis.

Physical Properties

Electrophoretic Mobility—In the Tiselius apparatus the mucolipide (Stage III) migrated with single, sharp ascending and descending boundaries, charged positively at pH 5.1 ($\mu = 8.96 \times 10^{-5}$ cm.^{2} per volt sec.) and charged negatively at pH 8.6 ($\mu = 10.6 \times 10^{-5}$ cm.^{2} per volt sec.).

Estimation of Molecular Weight—The mucolipide (Stage III) sedimented in the ultracentrifuge with a single sharp boundary in unbuffered 0.1 N $CaCl_2$ solution at pH 4.7 ($s_{20,w} = 12.1$ S), in 0.1 M citrate at pH 6.0 ($s_{20,w} = 10.2$ S) (Fig. 2), in 0.1 M trishydroxymethylaminomethane at pH 8.6

FIG. 2. Sedimentation pattern of the purified mucolipide (Stage III) in 0.1 M citrate buffer at pH 6.0. Pictures were taken at 8 minute intervals and proceed from left to right in time.

($s_{20,w} = 15.0$ S). The variation in the sedimentation values with pH is attributed to solvent interaction with the various ionic states of the lipide. The diffusion constant was estimated with a 1 per cent solution of the lipide in water at 4° and corrected to 20° (19). The value $D_{20,w} = 6.3 \times 10^{-7}$ cm.^{2} per sec. was obtained. The apparent partial specific volume was found as 0.74 cc. per gm. for two preparations. The calculated molecular weight, therefore, was 180,000.

Other Physical Properties—The purified mucolipide (Stage III) was a friable, glistening, pale yellow solid, crystalline in appearance but showing no orientation in polarized light; it had no characteristic absorption in ultraviolet light. It was freely soluble in water, giving non-viscous solutions, and in chloroform-methanol, moist benzene, methanol, and glacial acetic acid; it swelled without appreciable solution in pyridine at room temperature, was sparingly soluble in acetone, and was insoluble both in diethyl ether and in petroleum ether. The mucolipide, observed on an electrically heated stage, began to discolor at 180° and melted with decomposition between 245–250°. It was levorotatory: $[\alpha]_D^{25} -18°$ (in water).

Composition

Preliminary Observations—The mucolipide (Stage III) contained 3.8 per cent N, perhaps a trace of S, and less than 0.02 per cent P. The iodine number was 17. A 1 per cent solution in neutral water gave a pH reading of 4.7. The neutralization equivalent of the original preparation was 3810; equilibration with an excess of HCl in the cold, followed by exhaustive dialysis, lowered it to 1460. An electrometric titration curve showed a major region of inflection in the neighborhood of pH 6.5. Positive reactions were given for sialic acid (20) and hexose (21). The ninhydrin reaction (spot test on paper) also was positive. Mild acid hydrolysis (pH 1, 0.5 hour, steam bath) released sialic acid, small quantities of amino acid, and *N*-acetylhexosamine (22). More strenuous treatment (1 N HCl, 1 hour, steam bath) revealed hexose and produced humin. Vigorous hydrolysis (6 N HCl, 5 hours, steam bath) gave numerous amino acids and free hexosamine (22). Methanolysis (sealed tube, 10 per cent, v/v, aqueous concentrated HCl in methanol, 5 hours, steam bath) produced, on cooling, needles soluble in petroleum ether, sparingly soluble in 95 per cent ethanol, and giving a strongly positive hydroxamic acid reaction for esters. Removal of the esters with petroleum ether, followed by addition of alkali, permitted the extraction with diethyl ether of an organic base, hydrophobic, but soluble in most organic solvents, taking up bromine in glacial acetic acid which contained pyridine sulfate and reacting positively with ninhydrin. Each of the liberated entities was investigated individually.

Identification of Organic Base—The methanolysate, resulting from the treatment of 297 mg. of a relatively crude mucolipide preparation (Stage II) with 6 ml. of methanol and 0.5 ml. of aqueous concentrated HCl in a sealed tube for 3 hours on the steam bath, was subjected to six extractions, each time with 3 ml. of low boiling petroleum ether, in order to remove the fatty acid fraction described below. The methanolic layer was evaporated nearly to dryness in the flash evaporator, with the repeated addition of methanol and with NaOH pellets in the receiving flask. It was then brought to pH 8 with saturated $Ba(OH)_2$ solution and extracted seven times with a half volume of diethyl ether. The extract, concentrated to approximately 5 ml. in a stream of nitrogen, was washed with an equal volume of water and dried overnight with anhydrous sodium sulfate. The ether was blown off with nitrogen. The total crude product recovered from the ether solution was dissolved in a little 95 per cent ethanol and made just acid toward litmus by the addition of dilute alcoholic H_2SO_4. The chilled solution deposited an amorphous white sulfate which was employed for chromatographic studies, the procedures of which are described below. The sulfate of the base isolated from the mucolipide (Fig. 3) coincided in its migration with an authentic sample of sphingosine sulfate (23).

In four separate chromatographic systems a single component was observed in each case. For infrared analysis, a portion of the salt was crystallized from ethanol in the form of colorless needles. The infrared spectrum of the mucolipide base sulfate, pressed in KBr (Fig. 4), showed the same maxima as those previously published (24) for sphingosine sulfate in alcohol-free chloroform.

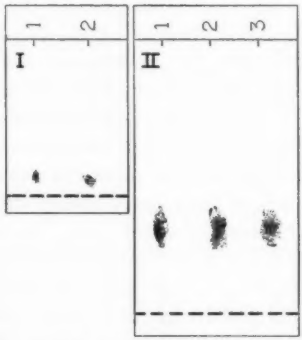

FIG. 3. Reversed phase partition chromatography of the base constituent of the mucolipide. I, Whatman paper No. 3 impregnated with silicic acid, irrigated with isopropanol-ammonia. (1) Mucolipide base; (2) authentic sphingosine. II, Whatman paper No. 54 impregnated with silicic acid, irrigated with butanol-ammonia. (1) Mucolipide base; (2) and (3) authentic sphingosine specimens.

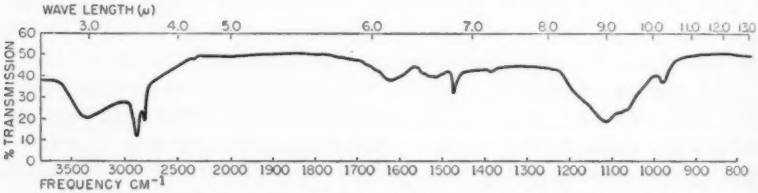

FIG. 4. Infrared spectrum of the mucolipide base sulfate, pressed in KBr

Fatty Acid Fraction—The petroleum ether extract of the methanolysate described above yielded the fatty acid methyl ester fraction. The iodine number was 21 and the saponification equivalent 350. The material was crystallized in approximately 60 per cent yield from anhydrous methanol containing 3 per cent low boiling petroleum ether. The first recrystallization gave colorless needles melting sharply at 57–58°; the melting point was not changed by subsequent crystallizations. Lignoceric acid methyl ester melts at 58.4° (25).

Identification of Hexose Components—Portions of the mucolipide of Stage II (5 mg. per ml.) were dissolved in increasing concentrations of dilute

sulfuric acid and heated for ½ hour in a boiling water bath. Humin was removed by filtration, the solutions were neutralized with $Ba(OH)_2$, and excess barium was removed with Amberlite IRC-50 (H). After centrifugation, the supernatant fluid was applied to paper chromatograms, each spot corresponding to 200 γ of mucolipide. A single hexose with the mobility of galactose was liberated in acid concentrations up to 2 N. At a 3 N acid concentration and above, a second hexose with the mobility of

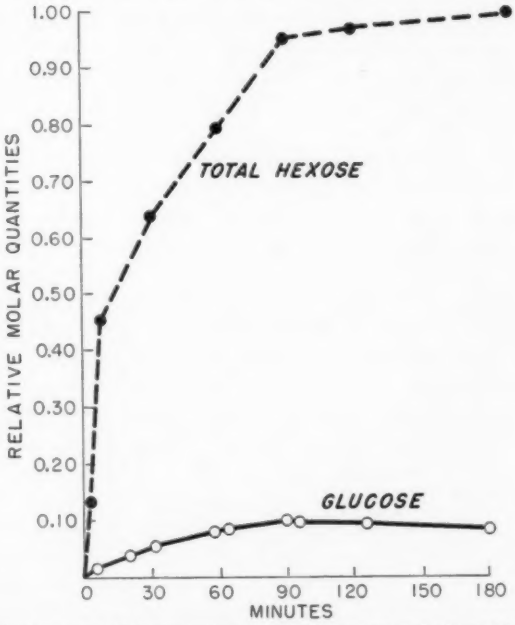

FIG. 5. Relative rates of hydrolytic release of glucose and of total hexose (glucose plus galactose) from the mucolipide by 3 N H_2SO_4 at 100°.

glucose was released in smaller quantity. Positive identification of the minor hexose component as glucose was made, on the chromatograms, with the aid of a glucose oxidase-peroxidase system, a modification of a published procedure being used (26). That galactose is initially liberated at a lower acid concentration than is glucose cannot be interpreted as indicative of a relative sequential arrangement of galactose and glucose in the lipide, since a careful study of hexose liberation in 3 N H_2SO_4 from a specially purified preparation showed that both hexoses are released at practically the same rate until complete hydrolysis of the glucose is effected (Fig. 5). For this experiment, a mucolipide preparation of Stage II was

subjected to countercurrent distribution, as described under "Amino acids," and then again to partition dialysis after being treated with $CaCl_2$.

Identification of Mucolipide Hexosamine—The hexosamine component of the mucolipide (Stage III) was completely liberated in 6 N HCl (sealed tube, steam bath) at the end of 4 hours. After removal of humin by filtration, the concentrated filtrate was applied to paper chromatograms (22) when a compound with the mobility of either glucosamine or galactosamine was observed. The hexosamine was degraded with ninhydrin to the next lower pentose homologue (27). A single pentose was observed which occupied the position of lyxose on chromatograms, indicating that the hexosamine was in all probability exclusively galactosamine. The degradation procedure used would, however, not distinguish between this amino sugar and talosamine.

Identification of Mucolipide Chromogen—The undegraded mucolipide (Stage III) was subjected to reaction with orcinol, hydrochloric acid, and ferric ion, *p*-dimethylaminobenzaldehyde and hydrochloric acid, and diphenylamine in acetic acid. The resulting spectra were practically identical with those given by ovine sialic acid (20). The chromogen was detached by 0.05 N sulfuric acid; paper chromatograms revealed the liberation of a component with a mobility corresponding in three separate systems to that of authentic ovine sialic acid, kindly donated by Professor G. Blix of Uppsala University.

For the isolation of the crystalline chromogen, a solution of 525 mg. of mucolipide (Stage II) at pH 2 (H_2SO_4) was heated for 1 hour at 80°. A flocculent precipitate, sedimented at 500 × *g* for 5 minutes, still contained considerable chromogen and was again heated for an additional 1.5 hours at pH 1.9. After removal of an insoluble residue, the clear supernatant fluids were neutralized with $Ba(OH)_2$ and Amberlite IRC-50 (H) as described above. The clear solution was permitted to dialyze for 48 hours in the cold, first against 50 and then against 25 ml. of distilled water. The dialysate was concentrated to 2 to 3 ml. in a flash evaporator, and the chromogen was precipitated as an amorphous gum with excess acetone. The precipitate contained not only chromogen, but some *N*-acetylhexosamine and amino acids. It was applied to a 0.8 × 25 cm. column of Dowex 1 (formate), 200 mesh, and washed, first with 100 ml. of distilled water and then with 40 ml. of 0.01 N formic acid. No chromogen was eluted. At 0.05 N HCOOH, chromogen began to appear; at 0.07 N HCOOH, the eluate gave a strong reaction for sialic acid. The eluates so reacting were combined and dried in a vacuum desiccator over NaOH and $CaCl_2$. The resulting light amber, glassy residue (32 mg.) was freed from color by precipitation with acetone from its solution in a little water. The chromogen crystallized in clusters of lancet-shaped needles at room

temperature from acetone containing 25 per cent (v/v) water and 1 per cent (v/v) low boiling petroleum ether. Some decomposition occurred during crystallization. The material was twice recrystallized, yielding approximately 4 mg. of colorless needles melting with decomposition at 183–185°. (Authentic ovine sialic acid melted at 184–186°, and a mixture of the two at 183–185°.) An infrared spectrum of our material is shown in Fig. 6; it corresponds in every detail to that of ovine sialic (*N*-acetylneuraminic) acid (28).

Amino Acids—The procedures for the hydrolysis of the mucolipide and the qualitative analysis of the amino acids by paper chromatography followed those given in a recent monograph (29). The solvent systems employed were 80 per cent aqueous phenol, and a mixture of 7.5 volumes *sec*-butyl alcohol, 1.5 volumes of 88 per cent formic acid, and 1 volume of water. Two-dimensional chromatography of 6 N HCl hydrolysates of the

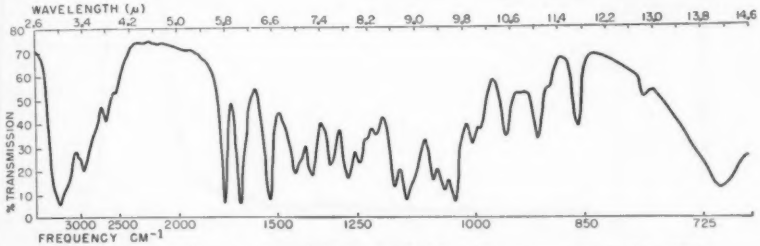

FIG. 6. Infrared spectrum of the mucolipide chromogen, pressed in KBr

mucolipide at all stages of purity revealed amino acids. Since this fraction comprised a relatively small part of the total weight of the molecule, special care was exercised in order to eliminate possible peptide or protein contaminants: (*a*) 10 mg. of mucolipide (Stage I), dissolved in 30 ml. of water, were blended for 5 minutes with 30 ml. of chloroform. The mixture was centrifuged at 1000 × *g* for 30 minutes, the clear aqueous supernatant layer was aspirated, without disturbing the interface, and lyophilized, yielding a residue of 9.2 mg. Hydrolysis in a sealed tube showed many amino acids on paper chromatograms. (*b*) 10 mg. of mucolipide (Stage II) were dissolved in 88 per cent (w/v) aqueous phenol, and 95 per cent ethanol was added until, at a concentration of 76 per cent ethanol (v/v), a distinct turbidity developed. The mucolipide was collected by centrifugation at 1000 × *g* for 1.5 hours at 4°, washed with ethanol, and dried under nitrogen. It was redissolved in 88 per cent phenol, and acetone was added until turbidity developed at 47 per cent acetone (v/v). The mucolipide was collected, washed, dried, and hydrolyzed as before. Paper chromatograms still showed numerous amino acids. (*c*) A solution of 100 mg. of mucolipide

(Stage I) in chloroform-methanol (1:1, v/v) was refluxed for 1 hour, cooled to −10° overnight, freed from a precipitate by filtration, and evaporated to dryness. A 10 mg. portion of the residue was dissolved in 300 ml. of glacial acetic acid at 60°; the solution was kept overnight at room temperature, filtered, and evaporated. The mucolipide residue still revealed numerous amino acids. (*d*) 10 mg. of the mucolipide (Stage II) were permitted to react with dinitrofluorobenzene in a modification (30) of Levy's arrangement (31). The reaction mixture was dialyzed exhaustively and lyophilized, and the residue extracted in turn with diethyl ether, ethyl

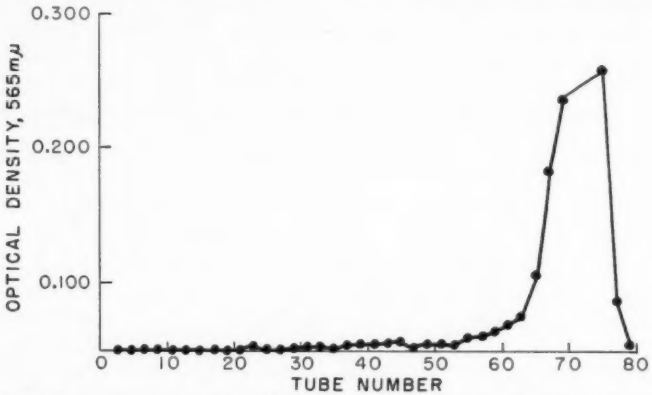

FIG. 7. Countercurrent distribution of the mucolipide (Stage II) between the phases formed on mixing carbon tetrachloride, methanol, and water. The plotted points are based on analyses of the upper phase for sialic acid by the direct Ehrlich reaction.

acetate, isoamyl alcohol, and acetone. The yellow mucolipide residue was hydrolyzed and found still to yield a number of free amino acids.

The combined evidence points to the occurrence of a peptide component as part of the mucolipide rather than as a contaminant. As a final measure of purification, the lipide was subjected to countercurrent distribution between the two phases formed on mixing carbon tetrachloride, methanol, and water (1.5:1.5:0.5, v/v/v). Countercurrent distribution of a relatively crude mucolipide preparation (Stage II) showed (Fig. 7) that the mucolipide moved rapidly in this system and that the procedure could be used to separate more slowly moving impurities. A purified lipide preparation (Stage III) was subjected to a 15 tube distribution. The tubes containing the mucolipide "peak" were combined, concentrated in the flash evaporator, and dried under nitrogen. This preparation is designated as "Stage IV." A 6 N HCl hydrolysate still revealed numerous amino acids.

The dinitrophenyl derivatives were formed, identified by two-dimensional paper chromatography, and the relative molar quantitites were estimated (30). The results are shown in Table I. It should be emphasized that these findings refer to a specific ox brain preparation; it remains to be seen whether the amino acid pattern is unchanged in different preparations.

The value listed as "proline" requires comment. The spot identified as dinitrophenylproline exhibited the two-dimensional mobility of the authentic proline derivative. Both derivatives exhibited absorption maxima at 385 mμ and had similar spectra, whereas the dinitrophenyl derivatives of other amino acids absorb maximally at 360 mμ. When, however, two-

TABLE I

Amino Acid Components of Mucolipide

Compound	Relative molar quantity
Glutamic acid	13
Glycine	10
Serine	8
Alanine	6
Threonine	4
Aspartic acid	3
Phenylalanine	2
Lysine	2
Arginine	1
Histidine	1
Valine	1
Cystine	Trace
"Proline"*	10

* Compare the text for a discussion of this component.

dimensional chromatograms of the free amino acids were developed with ninhydrin, a spot appeared at the position of proline that gave a blue-violet rather than the typical bright yellow color of the reaction product of proline with ninhydrin. Since sialic acid is known to be degraded eventually to α-carboxypyrrole (32), it is not impossible that the substance simulating the presence of proline actually represents an intermediate in the breakdown of sialic acid.

Hydrolysis Residue—After hydrolysis of the mucolipide (Stage II) at 90° (pH 1) for 3 hours, a residue amounting to 60 per cent of the starting material was obtained that had the solubility characteristics of a cerebroside and showed, after precipitation from anhydrous methanol, a sharp melting point at 180°. The hexose content (28 per cent) and the presence of galactosamine (3.9 per cent) and of amino acids indicated, however, that

the residue was not a cerebroside, but rather a cerebroside-like degradation product of the lipide.

Quantitative Estimation of Components and Balance

Table II presents a compilation of the various quantitative determinations carried out with the mucolipide. It will be seen that well over 90 per cent has been accounted for. The various techniques used will be detailed below.

TABLE II

*Composition of Mucolipide; Balance**

Component	Weight distribution	Molar distribution	No. of component molecules per mucolipide molecule
	per cent mucolipide	*μmoles per mg. mucolipide*	
Fatty acid	20.6	0.56	101
Sphingosine	16.5	0.55	99
Hexose†	23.0	1.3	234
N-Acetylgalactosamine	4.5	0.20	36
Sialic acid	26.0	0.84	151
Amino acid	4.4	0.32	58
Unbound water	2.6		
Total	97.6		
Correction‡	6.3		
Corrected total	91.3		

* The molecular weight of the mucolipide is taken as 180,000. For fatty acid the molecular weight of lignoceric acid, 368.6, was used. The average molecular weight of amino acid was computed from distribution studies similar to those summarized in Table I.

† The ratio of galactose to glucose is approximately 8:1 (compare Fig. 5).

‡ This correction allows for water eliminated by bonding.

One additional point deserves mention. The value of 0.32 μmole of amino acid per mg. of mucolipide was obtained by means of gasometric analysis. When the amino acid content was estimated in a hydrolysate (6 N HCl) by means of ninhydrin (33), the value, after correction for galactosamine and sphingosine, was considerably higher; namely, 1.5 μmoles per mg. of mucolipide. The hydrolysate yielded a steam-volatile base reacting with ninhydrin which was obtained as the hydrochloride in the form of yellowish hygroscopic needles. The compound, which has not yet been identified, migrated in the *sec*-butanol-formic acid-water system more slowly than ammonium ion or ethanolamine. Its solution in alkali gave the typical odor of lower amines. It cannot yet be decided whether this substance

represents an additional constituent of the mucolipide or a product of the decomposition of the previously identified constituents.

A mucolipide preparation of a purity corresponding to Stage IV was also analyzed for total acetyl content (Schwarzkopf Microanalytical Laboratories, Woodside, New York). The value found was 0.96 μmole of acetyl per mg. of mucolipide. This figure is in good agreement with the sum of the molar quantities of sialic acid, 0.84, and of *N*-acetylchondrosamine, 0.20 (Table II). The crystalline monoacetyl derivative obtained from the mucolipide, namely ovine sialic or *N*-acetylneuraminic acid, would, therefore, seem to represent the form in which this component actually occurs in the polymer. If the bovine form of sialic acid (diacetylneuraminic acid) (34) does occur in the mucolipide, the loss of a very labile *O*-acetyl group during the isolation of the polymer would have to be assumed.

Methodical Details

Iodine Number—This value was estimated by the procedure of Yasuda (35). When many analyses were required, it was found convenient to dilute the reaction mixture to a volume of 3 ml. with glacial acetic acid and to read the absorption of both the reaction mixtures and the standard bromine solution at 440 mμ in the spectrophotometer. The molar decrease in bromine concentration and, hence, the iodine number, could be readily calculated. The system obeys Beer's law within the range of the method and is quite reproducible.

Amino Acids—The total content of α-amino acids was estimated gasometrically (36).

Sphingosine—In each determination, 4 mg. of mucolipide were cleaved in a sealed tube with acidic methanol, and the liberated sphingosine was extracted quantitatively, as described above. Sphingosine, in the carefully washed extract, was estimated either by titration (Gilmont ultramicroburette) with bromophenol blue as indicator, or by estimation of the nitrogen content (Kjeldahl).

Phosphorus—The method of King (37) was employed.

Chromatographic Procedures—Free *amino acids* were made visible on the chromatogram (29) with the aid of a spray reagent consisting of a 0.2 per cent (w/v) solution of ninhydrin in *n*-butanol containing 5 per cent pyridine. The spots develop on standing ½ hour at room temperature.

The dinitrophenyl derivatives of the amino acids were prepared, chromatographed, and estimated quantitatively by the method mentioned before (30).

Sialic acid on Whatman No. 1 paper was irrigated (descending) with *sec*-butyl alcohol, acetone, acetic acid, water, 3:3:1.5:2.5 (38), $R_F = 0.50$;

sec-butyl alcohol, HCOOH, water, 75:15:10, R_F = 0.12; isobutyric acid, water, 5:3, adjusted to pH 3.5 to 3.9 with ammonia (39), R_F = 0.29. The spots were sprayed with orcinol, 0.5 per cent (w/v), and trichloroacetic acid, 15 per cent (w/v), in water-saturated *n*-butanol (18), followed by heating to approximately 120° for 5 to 10 minutes.

A clear separation of *glucose* and *galactose* was obtained on Whatman No. 2 paper of 56 cm. length, first washed with water and 95 per cent ethanol, by descending irrigation for approximately 20 hours with ethyl acetate, pyridine, water, 10:7:3, with the solvent being permitted to run off the paper. Under these conditions, galactose migrated about 25 cm. from the starting line; the ratio of the position of glucose to that of galactose was 1.1.

The glucose spots were identified by means of the following spray reagent: A solution of 5 mg. of peroxidase (Worthington Biochemical Corporation, Freehold, New Jersey) and 5 mg. of glucose oxidase (C. F. Boehringer and Sons, Mannheim, Germany) in 6 ml. of 0.1 M formate buffer, pH 3.9, was mixed with a solution of 10 mg. of *o*-tolidine in 4 ml. of acetone. The paper, after being dried in a stream of warm air, was sprayed lightly with the freshly prepared reagent. Glucose is demonstrated by the appearance within a few minutes at room temperature of bright blue spots stable for several hours. The buffer promotes stability of the blue color. This modification of a published procedure (26) has the advantage of being simpler and of preventing the diffusion and obliteration of small glucose spots during the spraying. The reagent is specific for glucose, with a sensitivity less than 1 γ of glucose per sq. cm. of chromatogram.

Lyxose and *arabinose*, as the ninhydrin degradation products of galactosamine and glucosamine, respectively, were separated on Whatman No. 1 paper either with the solvent used for the separation of glucose and galactose or with *n*-butanol, ethanol, water, 4:1:1, solvent front beyond the paper. Both hexose and pentose were sprayed with 0.5 per cent (w/v) 3,5-diiodosalicylic acid and 0.3 per cent (v/v) aniline in water-saturated *n*-butanol, followed by heating for approximately 5 minutes at 120–140°. The spots are stable indefinitely. Hexoses (tan to brown spots, yellow fluorescence under ultraviolet light) can be distinguished from pentoses (brick-red spots, brick-red fluorescence) and pentose 2-, 3-, or 5-phosphates (purple spots, pink fluorescence). The reagent was especially useful in detecting the pentoses in the presence of yellowish reaction products produced during the ninhydrin degradation of the hexosamines.

For free and *N-acetylhexosamines*, the previously mentioned conditions were used (22).

For the chromatography of *sphingosine* (2), the following systems were employed: (*a*) glass paper (Reeve Angel 934-AH), descending irrigation

with isopropanol, concentrated ammonia, water, 15:1:5 (v/v/v), R_F = 0.82; (*b*) Whatman paper No. 3 impregnated with silicic acid (40), same solvent, R_F = 0.91; (*c*) Whatman paper No. 54 impregnated with silicic acid, descending irrigation with *n*-butanol saturated with 2 N ammonia, R_F = 0.71; (*d*) Whatman paper No. 54 impregnated with an organosilicon compound (General Electric Dri-Film 9987), with the solvent as in (*c*) running beyond the edge of the paper. In all cases, the ninhydrin spray reagent described above was used.

Colorimetric Analyses—Sialic acid was estimated by the procedure of Werner and Odin (20), with suitable reduction in reagent volumes and quantities analyzed.

Fatty acids were estimated as the methyl esters which were quantitatively extracted after methanolysis in sealed tubes. Quantities ranging from 0.5 to 5 μmoles of methyl ester in petroleum ether were transferred to 15 ml. centrifuge tubes, and the solvent was evaporated under nitrogen. After the addition of 0.30 ml. of 2.5 per cent (w/v) hydroxylamine hydrochloride and of 0.40 ml. of 2.5 per cent (w/v) NaOH, both in 95 per cent ethanol, the mixture was heated for exactly 15 seconds by immersion in boiling water. It then was cooled for 5 minutes and mixed with 0.20 ml. of 2 N aqueous HCl and 4.00 ml. of 95 per cent ethanol. The addition of 0.50 ml. of 0.1 M $FeCl_3$ in 0.01 N HCl was followed by mixing, and the solution was kept at room temperature for 10 minutes. The resulting purple color was read at 535 mμ in the spectrophotometer against a reagent blank; solutions of methyl lignocerate, treated in the same manner, were used as the reference standard.

Total *hexose* was estimated by means of anthrone (21); free *hexosamine* by a modification of the Elson-Morgan procedure (41).

The problem of the reliable estimation of *glucose* in the presence of relatively large amounts of other hexoses is frequently encountered in the investigation of polysaccharides and related substances. For the present study, a specific system directly applicable to hydrolysates was developed; it was patterned after the procedure employed here for the demonstration of glucose on chromatograms. Directly before being used, 0.5 ml. of acetone containing 3 mg. of *o*-tolidine was mixed with 1.0 ml. of 0.05 M formate buffer (pH 3.9) containing 2 mg. of glucose oxidase and 1 mg. of peroxidase.[2] For the quantitative determination, 0.20 ml. of hydrolysate or of a stand-

[2] The glucose oxidase specimens used were obtained either from C. F. Boehringer and Sons, Mannheim, Germany, or by partial purification of the enzyme preparation marketed as "DeeO" (Takamine Laboratory, Clifton, New Jersey) through the removal of impurities that were insoluble in water or dialyzable. Horse-radish peroxidase was a preparation of the Worthington Biochemical Corporation, Freehold, New Jersey. The buffer strength was occasionally increased up to a 0.2 molarity if the solutions to be analyzed so required.

ard solution containing from 10 to 100 γ of glucose was mixed with 3.00 ml. of the formate buffer and with 0.20 ml. of the enzyme-tolidine reagent. After 20 minutes at room temperature, the blue color was evaluated at 630 mμ in the spectrophotometer against a reagent blank, with known amounts of aqueous glucose as standards for calculation. The molar extinction is approximately 6300; the color is stable for 10 minutes at room temperature and for several hours in an ice bath. After approximately 2 hours at room temperature, a complete transition occurs to a yellow color form (λ_{max} = 418 mμ) with approximately the same extinction and a stability of several hours. The addition of 0.1 ml. of 1:1 (v/v) isoamyl alcohol-concentrated hydrochloric acid gives an indefinitely stable purple color form (λ_{max} = 535 mμ) with approximately one-third the extinction of the blue or yellow forms. All three forms are reproducible and follow Beer's law in the range specified.

DISCUSSION

The mucolipide under discussion can be described as a highly polymerized ampholytic compound consisting of different quantities (in descending order) of galactose, sialic acid, fatty acid and sphingosine (these two in equal proportions), amino acid, *N*-acetylgalactosamine, and glucose. The molar proportions of the constituents listed in Table II make it obvious that we are dealing with an extremely complex substance for which no simple repeating unit should, for the time being, be put forward. Before the problem of bonding is discussed, a few other points should, however, be mentioned. The molecular weight is around 180,000, with no evidence of heterogeneity being observed. Of the constituents of the mucolipide, the two hexoses, the amino sugar and sialic acid, appear well characterized; the use of a specific enzyme provides unequivocal proof of the occurrence of small amounts of glucose. The presence of sphingosine has been demonstrated with reasonable certainty, though the occurrence of smaller amounts of dihydrosphingosine cannot yet be excluded. Owing to the lack of methods applicable to the small quantities available, the nature of the fatty acids has been ascertained less completely; a considerable proportion appears, however, to be represented by *n*-tetracosanoic (lignoceric) acid. In regard to the amino acid component, it would seem to occur as one polypeptide or as a series of several smaller ones. Despite multiple attempts to separate it from the lipide without hydrolysis, it persisted, and, although such statements cannot be made without reservation in the field of the lipides, we are inclined to consider the amino acids as an integral part of the mucolipide molecule. The distribution of the amino acids reveals an interesting pattern (Table I), but it has been pointed out that heretofore only one specimen has been fully analyzed in respect to the composition of its peptide constituent.

A detailed discussion of the arrangement of constituents is not yet advisable. The most obvious feature emerging from the inspection of the molecular proportions listed in Table II is the equimolarity of fatty acid and sphingosine. There is far from enough glucose (42) but more than enough galactose present to complete the 100 molecules of cerebroside per molecule of mucolipide that are indicated by the analytical results. This leaves 134 hexose molecules and all of the sialic acid, amino sugar, and peptide to be apportioned in such a way as to link 100 cerebroside molecules into a mixed polymer. Two properties of the mucolipide must be taken into account in this connection; namely, the low viscosity of its aqueous solution, which indicates a highly ramified structure, and its solubility in both water and organic solvents, which points to the existence of tracts with many hydroxyls separating the non-polar aliphatic chains. The simplest, but not necessarily correct, manner of viewing the structure would be as that of an assembly of units of different length and different degrees of branching, all terminating in cerebrosides, these units being held together through bifunctional cross-linking agents such as sialic or glutamic acid. It is significant that the mucolipide gives a positive hydroxamic acid test and that, whereas colorimetric analysis shows the presence of 0.84 μmole of sialic acid per mg., only 0.69 μ.eq. of acid per mg. can be demonstrated by titration. One may assume the functioning of cross-linking substances capable of forming both glycosidic and ester bonds. It will be remembered that the hydrolysis residue, which was free from sialic acid, still contained cerebroside, hexose, hexosamine, and amino acid.

It is, unfortunately, not yet possible to discuss the relationship, generic or specific, functional or metabolic, of the various forms in which cerebrosides occur in nervous tissue. Though the available figures (43) are not easily comparable, it would seem that the bulk of the cerebrosides occurs as monomers. But there is extant considerable evidence, mentioned in part above, of the occurrence of polymers comprising cerebrosides among their constituents. The mucolipide discussed here is, perhaps, the most complex representative of this class. Once the arrangement of its constituents is better understood, the multiple forms of sequential isomers in which such a polymer can exist will become obvious, as will also the interest attaching to the existence in brain of a macromolecular species having alternating regions of polarity and non-polarity as well as of ionizable groupings.

SUMMARY

The water-soluble lipide polymers of high molecular weight containing fatty acid, a sphingosine-like base, hexose, amino sugar, sialic acid or a related substance, and sometimes additional components such as peptides, are considered under the group designation of "mucolipide." The present

report outlines in detail the isolation of a complex ampholytic member of this group from ox brain by partition dialysis and its purification by repartition, salt dissociation, and countercurrent distribution, and describes its properties.

The purified ox brain mucolipide is homogeneous electrophoretically and ultracentrifugally, with a calculated molecular weight of 180,000. The constituents are apportioned as follows (number of component molecules per mucolipide molecule): fatty acid (mainly lignoceric), 101; sphingosine, 99; hexose (galactose and glucose in the ratio of 8:1), 234; ovine sialic (*N*-acetylneuraminic) acid, 151; *N*-acetylgalactosamine, 36; and amino acid, 58.

The structure, physical and chemical properties, and certain aspects of the biochemical significance of such an ampholytic lipide polymer are discussed.

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A "HEAT-ACTIVATED" DIPHOSPHOPYRIDINE NUCLEOTIDE PYROPHOSPHATASE FROM PROTEUS VULGARIS*

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(Received for publication, January 22, 1958)

Bodine *et al.* have described, in grasshopper eggs, a naturally occurring protyrosinase which is activated by many chemical agents and also by heating to 80° (1–4). Several "heat-activated" enzyme systems in bacteria have recently been described by Swartz *et al.* and by Kern and Natale (5–7). These enzymes have been found in *Proteus vulgaris* and *Mycobacterium butyricum*. In the former the enzymes were a DPN[1] pyrophosphatase and a 5′-nucleotidase, whereas in the latter the enzyme was a DPNase, hydrolytically cleaving DPN at the nicotinamide-ribose linkage (6). Both the *Proteus* enzymes and the *Mycobacterium* enzyme appeared to be in a completely inhibited form as isolated from extracts. Activation produced by boiling of extracts of these organisms was found to be related to destruction of a heat-labile protein inhibitor bound to a heat-stable enzyme. A dialyzable factor in extracts of *Proteus* or a factor in rabbit serum was found to be essential to protect the *Proteus* enzyme against denaturation by boiling. Inorganic pyrophosphate could also provide this protection. The *Mycobacterium* extracts differed from the *Proteus* extracts in having a much greater (12-fold) excess of free inhibitor.

The purpose of this paper is to describe the nature of the partially purified "heat-activated" DPN pyrophosphatase from *P. vulgaris* and also to define the products of the reaction.

* Contribution No. 225 of the McCollum-Pratt Institute, The Johns Hopkins University. Aided by grants from the National Science Foundation (NSF-G-2187) and the National Cancer Institute, National Institutes of Health (NIH-C-2374C).

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[1] The following abbreviations will be used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosine triphosphate; NMN, nicotinamide mononucleotide; 5′-AMP, 5′-adenylic acid; ADP, adenosine diphosphate; Versene, ethylenediaminetetraacetic acid; POP, inorganic pyrophosphate; Tris, tris(hydroxymethyl)aminomethane.

Materials

DPN of 90 per cent purity was obtained from the Pabst Brewing Company. Reduced DPN was prepared by enzymatic reduction (8) and TPN by enzymatic phosphorylation of DPN (9). ADP and ATP were obtained from the Sigma Chemical Company. Deamino-DPN was prepared chemically by deamination with nitrous acid and was isolated as the barium salt. Adenosine diphosphate ribose was prepared from DPN (10). The 3-acetylpyridine analogue of DPN was prepared by using the exchange reaction of the pig brain DPNase (11). 2′,5′-Diphosphoadenosine was prepared from the splitting of TPN by snake venom extract (12). Adenylic acid deaminase was prepared from rabbit muscle (13).

Methods

P. vulgaris X19 (ATCC 6380) cells were grown in large carboys in a minimal medium described by Lwoff and Querido (14). Vigorous aeration was carried out by using compressed air, with the air inflow being broken up by passage through a Dupont synthetic sponge. After incubation at 30° for 24 hours, the cells were harvested in a Sharples centrifuge and washed twice with 0.9 per cent KCl solution. The preparation of extracts and purification of the enzyme are described in a subsequent section.

A unit of enzyme activity is defined as that amount of enzyme which will cleave 1 μmole of DPN in 10 minutes at pH 7.5 at 37°. Protein determinations were performed by the method of Folin as modified by Lowry *et al.* (15).

DPN was determined by reduction with alcohol dehydrogenase of yeast. TPN was assayed by the use of isocitric dehydrogenase of pig heart (16).

Inorganic phosphate was measured by a modification of the procedure of Fiske and Subbarow (17). Chromatographic identification of 2′,5′-diphosphoadenosine was by the method of Wang *et al.* (18). Monoester phosphate was determined by using prostatic monoesterase prepared by the procedure of Markham and Smith (19).

Results

Effect of Heat—Crude extracts of this organism, prepared by sonic oscillation, do not hydrolyze DPN at any appreciable rate. Fig. 1 shows that this extract could be rendered active by exposure to a boiling water bath for 2 minutes. Although the enzyme is remarkably heat-stable, this stability is not absolute; *i.e.* after 15 minutes of boiling, the activity is reduced by 75 per cent (Table I). Maximal activation is achieved by

boiling for 2 minutes. Long exposure of enzyme to temperatures below 100° will produce only partial activation.

Although crude enzyme preparations can withstand extremely high temperatures, the optimal temperature of incubation for this reaction is

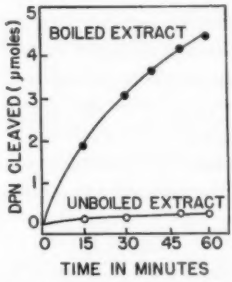

FIG. 1. Effect of heat. A 2 ml. aliquot of the crude sonic extract was kept in a boiling water bath for 2 minutes and then immediately cooled in an ice bath and tested for activity as follows: The reaction mixture at 37° contained 0.5 ml. of enzyme, 1.3 ml. of 0.2 M Tris buffer (pH 7.5), and 4.5 μmoles of DPN in a final volume of 3.0 ml. Aliquots (0.3 ml.) of the reaction mixture were removed at the times indicated, and the DPN present was assayed in a Beckman spectrophotometer by the alcohol dehydrogenase method.

TABLE I

Heat Resistance of Pyrophosphatase

The crude enzyme preparation was heated at 100° for the times noted. Then 0.5 ml. of the enzyme was incubated with 5 μmoles of DPN in 0.1 M Tris buffer at pH 7.5. The final volume was 3.0 ml. DPN remaining after 25 minutes was assayed by the alcohol dehydrogenase method after removal of appropriate aliquots.

Time	Per cent maximal activity
min.	
1	88
2	100
7	65
10	48
15	25

only 50°. The Q_{10} for the range 30–40° is 1.75. Preparations that have been boiled and then assayed at 70° show no activity. However, it can be shown that this lack of activity is related to a reversible heat denaturation of the enzyme (Table II). Boiled preparations that are inactive on assay at 70° can be restored to full activity by chilling and then allowing the reaction to proceed at a lower temperature (37°).

Heat Stability Factors for Enzyme—Crude enzyme preparations are stable to boiling in aqueous solution. Dialysis of crude *Proteus* extracts for 18 hours against distilled water produces an enzyme that is totally inactive both before and after heating to 100°. Dialysis of similar extracts subsequent to boiling yields a preparation with 75 per cent of full activity. At least 70 per cent of full activity can be attained by adding a 1.0 M HCl filtrate of a *Proteus* extract to the dialyzed enzyme before boiling. A similar addition after the enzyme has been boiled is not effective in reactivating the enzyme. In Fig. 2 it can be seen that two factors are needed for full activation of the crude dialyzed preparations. Inorganic pyrophosphate (6 μmoles per ml.) will replace the naturally occurring factor which stabilizes the enzyme to heat. Pyrophosphate addition to the dialyzed extract

TABLE II

Reversible Heat Denaturation

The enzyme used was a crude sonic extract of *P. vulgaris* that had been boiled for 2 minutes. The incubation mixture consisted of 0.2 ml. of enzyme, 3 μmoles of DPN, and 0.1 ml. of 0.1 M phosphate buffer (pH 7.5). The final volume was 1.5 ml. Aliquots taken at 10 minutes and 20 minutes. Mixture I incubated at 37° for the entire 20 minutes. Mixture II incubated at 70° for the first 10 minutes, then rapidly chilled and incubated at 37° for the next 10 minutes.

Time	μmole DPN cleaved	
	Mixture I (37°)	Mixture II (70°, then 37°)
min.		
0–10	0.98	0
10–20	0.84	0.98

after boiling will not yield any enzymatic activity. A large number of organic phosphates or pyrophosphates (possible substrates or products of this enzyme's activity such as DPN, TPN, ATP, NMN, 5′-AMP, etc.) will not replace inorganic pyrophosphate in its "stabilizing" role. There is, however, a factor, inactivated by trypsin and by dialysis, from rabbit plasma which will replace pyrophosphate. The second factor required for full activity of the dialyzed preparation is Co^{++}. This is needed only for the enzymatic reaction and is added to the incubation mixture containing previously boiled enzyme.

Purified enzyme preparations (free of bound inhibitor) are also protected against high temperatures by sodium pyrophosphate (Table III). It can be seen that the concentration of pyrophosphate (0.00026 M) needed for maximal protection of the purified enzyme is only about one-thirtieth the concentration required for protection of the crude dialyzed enzyme (0.006 M). There is actually a diphasic effect with pyrophosphate. With concentra-

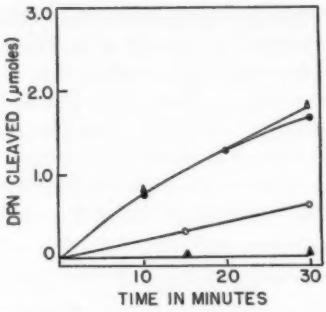

FIG. 2. Factors required by dialyzed extracts. Crude sonic extract was dialyzed for 18 hours against distilled water and then boiled. When sodium pyrophosphate was used, it was added to the extract prior to boiling (6 μmoles of POP per ml. of extract). When $CoCl_2$ was added, the final concentration was 0.0013 M. The reaction mixture at 37° consisted of 0.5 ml. of crude enzyme preparation, 0.65 ml. of 0.2 M Tris buffer (pH 7.5), and 2.7 μmoles of DPN in a final volume of 1.5 ml. Aliquots (0.3 ml.) of the reaction mixture were removed at the times indicated, and the DPN present was assayed in a Beckman spectrophotometer by the alcohol dehydrogenase method. △ = boiled, undialyzed extract; ● = boiled, dialyzed extract plus POP plus Co^{++}; ○ = boiled, dialyzed extract plus POP; ▲ = boiled, dialyzed extract plus Co^{++}.

TABLE III

Protective Effect of Sodium Pyrophosphate for Purified Enzyme

The enzyme preparation was purified 40-fold and was completely free of inhibitor. The enzyme was boiled for 2 minutes in the presence of varying concentrations of inorganic pyrophosphate, then it was rapidly cooled. The ability of these boiled preparations to cleave DPN was compared to that of unboiled purified enzyme without added pyrophosphate. The incubation mixture contained 3 μmoles of DPN, 0.02 ml. of boiled enzyme, 0.07 M Tris buffer (pH 7.5), and 0.0007 M $CoCl_2$. The final volume was 1.5 ml. DPN was measured by the alcohol dehydrogenase method.

POP concentration of boiled enzyme	Per cent of activity of unboiled active enzyme
M	
1×10^{-1}	0
1×10^{-2}	25
7×10^{-3}	34
5×10^{-3}	45
3.3×10^{-3}	73
6.6×10^{-4}	85
3.3×10^{-4}	92
2.6×10^{-4}	105
1.3×10^{-4}	93
6.6×10^{-5}	23
0	0

tions above 0.00026 M there is a reduction in demonstrable enzymatic activity. At these concentrations the amount of pyrophosphate carried over to the assay system with the enzyme preparation may be sufficient to exert a chelating effect on the cobalt. Thus, when the 40-fold purified enzyme preparation, free of inhibitor (Table III), has been boiled in 0.1 M sodium pyrophosphate for 2 minutes, it is completely inactive on subsequent assay. However, in the assay system the final concentration of pyrophosphate carried over with enzyme is as high as 0.0013 M. It should be pointed out,

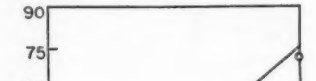

FIG. 3

FIG. 4

FIG. 3. Effect of varying inhibitor concentration. The reaction mixture contained 0.3 ml. of an 18-fold purified enzyme preparation (enzyme-inhibitor complex), 2 μmoles of DPN, 0.65 ml. of 0.2 M Tris buffer at pH 7.5, and 1.0 μmole of $CoCl_2$ in a final volume of 1.6 ml. Prior to incubation the enzyme had been boiled with sodium pyrophosphate (0.006 M) for 2 minutes. The inhibitor fraction consisted of crude *P. vulgaris* sonic extract that had been dialyzed for 18 hours against distilled H_2O. DPN was assayed by the alcohol dehydrogenase method.

FIG. 4. Effect of chelating agents. The incubation mixtures consisted of 0.5 ml. of crude enzyme preparation, 1.3 ml. of 0.2 M Tris buffer (pH 7.5), and 5 μmoles of DPN in final volume of 3.0 ml. DPN was measured by the alcohol dehydrogenase method. △ = Tris buffer used; ○ = sodium pyrophosphate buffer (0.09 M final concentration); ● = 0.2 M Tris buffer plus Versene (0.0013 M final concentration).

nevertheless, that the range of pyrophosphate concentration which inhibits the enzyme in the presence of a fixed Co^{++} concentration varies considerably according to the degree and method of purification of the enzyme.

Inhibitor—The presence of an inhibitor can be shown conveniently in crude extracts by mixing boiled purified enzyme with unboiled extract, used as a source of inhibitor. Fig. 3 shows a linear relationship between amount of inhibitor and per cent inhibition. In crude *Proteus* extracts there is only a small amount of free, *i.e.* unbound, inhibitor. Thus, 0.3 ml. of the dialyzed extract will produce about 60 per cent inhibition of 0.3 ml. of boiled enzyme preparation. The inhibitor has been purified thus far only several fold by alkaline ammonium sulfate (50 to 80 per cent)

fractionation and subsequent ethanol precipitation (40 to 55 per cent). It is resistant to ribonuclease action and to the action of crude pancreatic protease preparations. It can be irreversibly inactivated on acidification to pH 2.0 at room temperature for 10 minutes. This same acidification procedure will not inactivate the enzyme. Thus, this procedure may be used in place of heating in order to "activate" the enzyme fully. On neutralization the inhibitor remains inactive. Attempts to dissociate the inhibitor from the enzyme by using NaCl of various ionic strengths have been unsuccessful. Dialysis of extracts of *Proteus* against chelating agents such as cyanide, Versene, and pyrophosphate has not removed the inhibitor.

Metal Requirement—Chelating agents such as Versene or inorganic pyrophosphate in high concentrations (0.09 M) in the reaction mixture can produce almost complete inhibition of the active boiled enzyme (Fig. 4). A much lower pyrophosphate concentration (0.005 to 0.006 M) is needed to protect the enzyme against denaturation on boiling. A metal ion, Co^{++}, is needed for the enzymatic reaction itself and need not be added to the enzyme before it is boiled (Fig. 2). A large number of cations, including Mg^{++}, Ca^{++}, Zn^{++}, Fe^{++}, Na^{+}, K^{+}, *etc.*, were tested, and of these only Mn^{++} could partially replace Co^{++}. The latter is optimally effective at 10^{-4} M concentration, but a significant effect can be seen at a concentration of 6×10^{-6} M. Certain cations such as Ca^{++}, Zn^{++}, and Cu^{++} inhibit the Co^{++} effect.

Purification Procedure for Enzyme-Inhibitor Complex

Step 1. Preparation of Crude Extract—To each 3 gm. (wet weight) of cells were added 18 ml. of distilled water, and the suspension was placed in a Raytheon sonic disintegrator at approximately 9000 kc. for 30 minutes. The resulting slightly turbid solution served as the crude extract. Only after prolonged centrifugation at high speeds was any sedimentable cell debris obtained. Repeated freezing and thawing led to moderate loss of activity. However, the enzyme could be kept in the cold room for several weeks without loss of activity.

Step 2. Treatment with Protamine Sulfate—To each 210 ml. of crude extract were added 420 ml. of 0.04 M Tris (pH 7.5) containing 0.2 per cent protamine sulfate. After standing in the cold for 1 hour, the precipitate was removed by centrifugation. Approximately 560 ml. of clear supernatant fluid were obtained.

Step 3. Acetone Fractionation—65 ml. of cold acetone were slowly added to each 100 ml. of supernatant fluid obtained from Step 2. The enzyme preparation was kept at $-5°$ to $-10°$ in a dry ice-acetone bath. The precipitate was removed by centrifugation at 20,000 $\times$ *g* for 10 minutes

and discarded. To the supernatant fluid another 55 ml. of cold acetone were slowly added with stirring. The supernatant fluid was discarded after centrifugation at 20,000 × *g* for 10 minutes. The precipitate was dissolved in 100 ml. of demineralized H_2O. The solution was somewhat turbid, and the undissolved material was removed by centrifugation.

Step 4. Treatment with Calcium Phosphate Gel—To each 85 ml. (pH = approximately 6.0) from Step 3 were added 10 ml. of aged calcium phosphate gel (20.8 mg. per ml.). The mixture was gently stirred for 30 minutes. After centrifugation, the gel was saved for Step 5.

Step 5. Elution of Enzyme from Calcium Phosphate Gel—To the gel from Step 4 46 ml. of 0.01 M potassium phosphate buffer (pH 6.5) were

TABLE IV

Purification Procedure; Enzyme-Inhibitor Complex

See the text for a description of the procedure.

Step No.	Units per ml.	Total units	Units per mg. protein
Step 1 (crude extract)	13.00	2730	0.97
" 2	5.83	3265*	1.98
" 3	4.50	2520	4.05
" 4			
" 5	3.10	1729	9.40
" 6	2.30	1169	15.5
" 7	2.30	1030	19.1

* The increase in total units after Step 2 of purification may have been related to incomplete destruction of inhibitor by boiling of the crude extracts of Step 1 (during the assay) or related to removal of another inhibitory factor by the protamine step.

slowly added with trituration. The mixture was thoroughly stirred and centrifuged, and the supernatant fluid was saved. The gel was then rewashed with 37 ml. of phosphate buffer, and the mixture was centrifuged. The two supernatant fluids were pooled (volume of 83 ml.).

Step 6. Treatment with Alumina Cγ—To each 67 ml. of eluate from Step 5 was added 1.0 ml. of aged alumina Cγ gel (15 to 20 mg. per ml.). The mixture was stirred for 30 minutes. The gel was then centrifuged, and the clear supernatant fluid was saved.

Step 7. Passage through Column of Whatman No. 5 Paper—0.5 gm. of Whatman No. 5 filter paper was macerated in distilled water in a Waring blendor and then placed in a small column approximately 1.5 cm. in diameter. The filter paper was packed tightly. 25 ml. of enzyme solution were passed through the column, and the eluate was collected. Table IV summarizes the purification of enzyme-inhibitor complex.

Purification Procedure for Free Enzyme

Step 1. Preparation of Crude Extract—This method was the same as Step 1 above.

Step 2. Treatment with Protamine Sulfate—This procedure was the same as Step 2 above.

Step 3. Boiling Procedure—To 132 ml. of protamine supernatant fluid were added 4.0 ml. of 0.2 M sodium pyrophosphate (pH 7.5). The solution was poured into a wide bottomed Erlenmeyer flask and placed in a boiling water bath for 3 minutes. The solution was then rapidly cooled by placing the flask in ice. The precipitate was not removed.

Step 4. Acetone Fractionation—90 ml. of chilled acetone were slowly added with stirring to the boiled enzyme of Step 3. The precipitate was removed by centrifugation at 20,000 × *g* for 15 minutes. To the supernatant fluid were added another 74 ml. of acetone. The resulting precipitate was removed by centrifugation after the solution had stood in the cold for 30 minutes. The precipitate was then dissolved in 136 ml. of distilled water. Insoluble material was removed by centrifugation.

TABLE V

Purification Procedure of Active Enzyme

See the text for a description of the procedure.

Step No.	Units per ml.	Total units	Units per mg. protein
Step 1 (crude extract)	13.00	2730	0.97
" 2	5.83	3265*	1.98
" 3			
" 4	2.80	1615	
" 5	1.27	710	37.4

* See the same footnote in Table IV.

Step 5. Treatment with Calcium Phosphate Gel—To 135 ml. of the enzyme preparation from Step 4 were added 18.2 ml. of aged calcium phosphate gel (20.8 mg. per ml.). The mixture was stirred slowly for 1 hour. The gel was removed by centrifugation, and the supernatant fluid was discarded. The enzyme was eluted from the gel by addition with stirring of 132 ml. of 0.01 M potassium phosphate solution (pH 6.5) and subsequent removal of gel by centrifugation. Table V summarizes the purification of the free enzyme.

Specificity of Enzyme—DPN and DPNH were both cleaved quite rapidly by the enzyme. TPN, however, was destroyed at only 8 per cent the rate of DPN splitting. Analogues of DPN such as the 3-acetylpyridine analogue and deamino-DPN were split at rates comparable to the splitting

of DPN itself. Adenosine diphosphate ribose, ADP, and ATP were also cleaved as was inorganic pyrophosphate. Thiamine pyrophosphate and trimetaphosphate were not split by the enzyme.

Products of Reaction—The pyrophosphatase could not be completely freed from a contaminating 5′-nucleotidase in any of these preparations. The latter enzyme was also heat-activated, requiring inorganic pyrophosphate or the plasma factor for stability during boiling, but did not require Co^{++} for activity. Because of the presence of this 5′-nucleotidase, the stoichiometry of the reaction was such that for every micromole of DPN cleaved 1 μmole of adenosine, 1 μmole of nicotinamide riboside, and 2 μmoles of inorganic phosphate appeared. The reaction went to completion. There was no evidence that this enzyme would synthesize DPN by using ATP and NMN. Attempts to demonstrate the presence of free adenylic acid as an intermediate, by utilizing the adenylic acid deaminase of muscle, were unsuccessful.

In order to demonstrate that the point of cleavage by the enzyme was at the pyrophosphate linkage, the products of the cleavage of TPN were investigated, since it was found that the 5′-nucleotidase of the *Proteus* did not attack the 5′-phosphate grouping of 2′,5′-diphosphoadenosine.

47.9 μmoles of TPN were incubated for 22 hours with large amounts of purified active enzyme. At the end of this time 46 μmoles had been cleaved, as measured by TPN-specific isocitric dehydrogenase. The products of the reaction were separated on a Dowex 1 formate form column. 35 μmoles of nicotinamide riboside could be found in the water eluate as measured by the absorption at 325 mμ on the addition of molar KCN. A total of 45 μmoles of adenine (or adenine derivatives) was present in the water eluates and in the washings with 0.1 M formic acid-formate and 0.02 to 0.05 M sodium nitrate-nitric acid. The bulk of the adenine-containing material (30.4 μmoles) had extremely acid properties and could only be eluted by prolonged washing with 0.05 M sodium nitrate-nitric acid. This compound was shown to contain two phosphate groups per adenine moiety. By utilizing prostatic monoesterase, it was shown that both phosphates were of the monoester type.

The acid compound, containing one adenine and two monoester phosphate groups, which had been eluted from Dowex 1 by 0.05 N sodium nitrate-nitric acid, was chromatographed on Whatman No. 3 paper in Carter's Solvent System (20) of 5 per cent Na_2HPO_4 and isoamyl alcohol. This compound was identified by its ultraviolet light quench with an R_F of 0.89. Authentic 2′,5′-diphosphoadenosine isolated after snake venom pyrophosphatase action on TPN had an R_F of 0.88; this therefore can be taken as conclusive evidence that the *Proteus* enzyme is a pyrophosphatase.

Effect of pH on Activity—The pH optimum for the enzyme is 7.5. The

activity rapidly decreases on the acid side of this point, being only 30 per cent at pH 6.5 and 5 per cent at pH 5.5. There is only a gradual drop in activity when the pH is shifted to the alkaline side of pH 7.5. Over 65 per cent of the maximal activity is present at pH 10.5.

Absence of Other "Heat-Activated" Enzymes—The "heat-activated" pyrophosphatase and 5′-nucleotidase were the only "heat-activated" enzymes found in crude extracts of *P. vulgaris*. Non-specific phosphatase, alcohol dehydrogenase, and nucleoside phosphorylase are present in unheated extracts and absent or greatly diminished after 2 minutes of boiling. Aryl sulfatase activity is absent in the unheated as well as in the boiled extracts.

DISCUSSION

The pyrophosphatase of *P. vulgaris* can be demonstrated in extracts of this organism only after inactivation, by boiling or acid treatment, of a bound inhibitor. The inhibitor is probably a protein and is quite tightly bound to the enzyme. The pyrophosphatase is quite heat-stable and is similar in this regard to trypsin, myokinase, and ribonuclease (21–23). Like trypsin it can be shown to undergo reversible heat denaturation (21).

The evidence that this heat-stable pyrophosphatase is a protein stems from the following observations: It is non-dialyzable, it has a temperature optimum for activity, it is precipitable by acetone and can be adsorbed on calcium phosphate and alumina gels, and it is inactivated on exposure to pepsin at pH 2.0.

Similar inhibitor-enzyme relationships have been found in other microorganisms (5–7). This points up the fact that an enzyme may appear to be absent in a given tissue, solely due to being bound to an inhibitor derived from that same tissue. Thus, some "manipulation" (boiling, acid treatment, etc.) may be required to demonstrate the presence of this enzyme.

The stability of this enzyme protein to heat denaturation is dependent on inorganic pyrophosphate or on certain, as yet poorly defined, factors in serum or extracts of *P. vulgaris*. Inorganic pyrophosphate is one of the substrates of this enzyme and might protect the active site of the enzyme against denaturation much as glucose protects hexokinase against inactivation by trypsin (24). However, none of the other substrates for this enzyme affords any protection. The similar protection by pyrophosphate of the heat-activated 5′-nucleotidase of *Proteus* suggests that the above postulate is incorrect.

Because of the presence of a contaminating non-specific 5′-nucleotidase, it has not been possible to show directly the primary products of the splitting of DPN. By utilizing the information that the 5′-nucleotidases from

snake venom and bovine seminal fluid do not liberate the 5′ grouping from the diphosphoadenosine fragment of TPN (25), and that TPN was split only slowly by this *Proteus* pyrophosphatase, an attempt was made to demonstrate the primary products of enzymatic action. 2′,5′-Diphosphoadenosine and nicotinamide riboside were the main products isolated, strongly supporting the postulated pyrophosphatase nature of the enzyme.

The reaction between the DPN pyrophosphatase and its inhibitor is unlike that of trypsin and its pancreatic inhibitor in that, although both complexes are essentially undissociable at neutral pH, only the trypsin-pancreatic inhibitor compound is reversibly dissociable at pH 1.0 to 2.0 (26). Incubation of the *P. vulgaris* crude extract at pH 2.0 for 10 minutes at 25° yields active enzyme. If, at the end of this time, the extract is neutralized, the preparation remains active without boiling.

Whether the DPN pyrophosphatase is present in the "inhibited form" in the bacterial cell cannot be definitely determined. However, the fact that whole cells can cleave DPN at the pyrophosphate bond suggests that inhibitor and enzyme are brought together by cell disruption. This is in contrast to the "heat-activated" DPNase in *M. butyricum*. In that organism, whole cells do not cleave DPN (6).

The inhibitor protein in *Proteus* appears to be under some form of nutritional control. When this organism is grown on a medium containing yeast extract and tryptone, essentially no inhibitor can be found (5).

SUMMARY

1. A heat-activated enzyme from *Proteus vulgaris* has been described. This enzyme is a pyrophosphatase splitting diphosphopyridine nucleotide, triphosphopyridine nucleotide, and several other organic pyrophosphate compounds. The enzyme has been purified approximately 40-fold. Cobalt is needed for activity of the purified enzyme.

2. This enzyme cannot be demonstrated in crude extracts of the organism because of the presence of a heat-labile protein inhibitor which is bound to the enzyme. There is an approximately 60 per cent excess of free inhibitor in crude extracts.

3. The enzyme has a temperature optimum at 50° and undergoes reversible heat denaturation at higher temperatures.

4. The heat stability of the enzyme is dependent on certain cofactors in serum, in extracts of *P. vulgaris*, or on inorganic pyrophosphate.

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FORMATION OF A CYCLIC ADENINE RIBONUCLEOTIDE BY TISSUE PARTICLES*

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(Received for publication, October 23, 1957)

Studies in this laboratory have shown that the increased formation of phosphorylase in liver homogenates observed in the presence of epinephrine and glucagon was mediated by a heat-stable factor (1). This factor accumulated when the hormones were incubated with adenosine triphosphate and particulate fractions of liver homogenates and stimulated phosphorylase formation in supernatant fractions in which the hormones had no effect (1).

The factor has been isolated and characterized, as detailed in Sutherland and Rall (2), and proved to be a cyclic adenine ribonucleotide (2, 3), identical to a compound originally designated cyclic dianhydrodiadenylic acid by Cook, Lipkin, and Markham (4), and recently shown to be the mononucleotide adenosine-3′,5′-phosphoric acid.[1] This report is concerned with the conditions for the formation of cyclic 3,5-AMP.[2] It was found that particulate preparations not only from liver, but also from heart, skeletal muscle, and brain, produced this compound. In a C^{14} tracer experiment it was determined that ATP-8-C^{14} added to the incubation mixtures gave rise to cyclic 3,5-AMP with no apparent dilution of the radioactivity.

Methods

Preparation of Particulate Fractions and Homogenates—Washed particles from preincubated liver slices were prepared as described previously (1),

* This investigation was supported in part by a research grant (No. H-2745) from the National Heart Institute of the United States Public Health Service.

[1] In a personal communication, Professor David Lipkin has informed us that a molecular weight determination and other data prove that the compound is adenosine-3′,5′-phosphoric acid. The complete proof of structure of the nucleotide will be presented in a forthcoming publication by D. Lipkin, R. Markham, and W. H. Cook.

[2] The following abbreviations are used: cyclic 3,5-AMP, adenosine-3′,5′-phosphoric acid; 5′-AMP, adenosine 5′-phosphate; 2′-AMP, adenosine 2′-phosphate; 3′-AMP, adenosine 3′-phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; LP, liver phosphorylase; dephospho-LP, liver dephosphophosphorylase; LP phosphatase, liver phosphorylase phosphatase; pApA, 5′-phosphoadenosine-3′-adenosine 5′-phosphate; pApApA, 5′-phosphoadenosine-3′-adenosine-5′-phosphoadenosine-3′-adenosine 5′-phosphate.

by incubation of dog liver slices at 37° for 15 minutes, homogenization in an all-glass homogenizer, and collection and washing of the particles by centrifugation at 1200 × *g*. For the bulk of the experiments reported here, including large scale preparation of cyclic 3,5-AMP, particles were prepared from liver slices not previously incubated. Perfused and chilled dog liver (300 to 400 gm.) was rapidly sliced into pieces approximately 5 × 25 × 50 mm., rinsed in 3 volumes of cold 0.33 M sucrose, and homogenized for 15 to 20 seconds in 2 volumes of 0.33 M sucrose in a Waring blendor. The homogenates were strained through a single layer of gauze and centrifuged for 17 minutes at 2000 × *g* on the International centrifuge (No. 3) at 5°. The supernatant fluid was removed by aspiration, and the precipitate was resuspended to the original volume of the homogenate with 0.25 M sucrose. The washed particles were collected by centrifugation as before and were suspended in an equal volume of 0.25 M sucrose. The yield of washed particles averaged about 140 ml. of suspension (about 70 ml. of packed volume) per 100 gm. of liver.

For some experiments, the precipitate collected at 2000 × *g* was washed with distilled water instead of 0.25 M sucrose. The particles were suspended in 3 volumes of cold glass-distilled water, and the suspension was allowed to stand at 2° for 20 minutes before being centrifuged 15 minutes at 5000 × *g* on the Servall SS-1 centrifuge. The resulting loosely packed precipitate was suspended in one-third its volume of 0.5 M sucrose.

The preparation of washed particles from heart, skeletal muscle, and brain was similar to that from liver. The tissues were removed as rapidly as possible from a mature dog, chilled in 0.33 M sucrose, sliced, and homogenized in 4 volumes of 0.33 M sucrose in a Waring blendor. The homogenates were centrifuged 15 minutes at 2000 × *g* in the Lourdes SL centrifuge. The precipitate was washed four times by suspension in 0.25 M sucrose and centrifuged for 15 minutes at 4000 × *g*. The final precipitate was suspended in an equal volume of 0.25 M sucrose.

Preparation of Heated Extracts Containing Cyclic 3,5-AMP—The complete system finally adopted for the production of cyclic 3,5-AMP contained 0.04 M Tris (pH 7.5),[3] 2.5 × 10^{-3} M $MgSO_4$, 2 × 10^{-3} M ATP, 6.67 × 10^{-3} M caffeine, 0.01 M NaF, 5.5 γ per ml. of epinephrine, 1 γ per ml. of crystalline glucagon, 1.0 ml. of 0.25 M sucrose, and 1.0 ml. of washed particle suspension per 2.4 ml. of incubation mixture. The final concentration of sucrose was approximately 0.2 M. For small scale experiments, the incubation mixtures at a final volume of 2.4 to 3.6 ml. were shaken in air in a Dubnoff metabolic incubator at 30°. At various time intervals,

[3] Concentrations stated here and in Tables I to IV and Figs. 1 and 2 represent final concentrations in the incubation mixtures.

1 ml. samples were transferred to culture tubes and heated in boiling water for 3 minutes and chilled. For the large scale preparation of cyclic 3,5-AMP, the incubation mixtures were stirred at 30° in an atmosphere of 100 per cent oxygen and then heated in boiling water for 5 to 7 minutes. After removal of insoluble material by centrifugation, the heated extracts were stored at −20° if not used immediately.

Assay of Cyclic 3,5-AMP—A mixture containing 10.9 μmoles of Tris buffer (pH 7.5), 0.68 μmole of $MgSO_4$, 0.54 μmole of ATP, and 0.25 μmole of caffeine was pipetted into a 15 × 125 mm. culture tube, and either unknown, standard, or suitable blank solution was added to obtain a final volume of 0.1 ml. Then, 0.2 ml. of an 11,000 × *g* supernatant fraction of a liver homogenate, diluted 10- to 20-fold in 0.25 M sucrose and fortified with 1.2 to 1.8 units of dephospho-LP, was added; this mixture was incubated at 30° to allow the formation of LP to occur. After 10 minutes, 2.8 ml. of the phosphorylase assay reagent mixture, containing glucose 1-phosphate, glycogen, and 5′-AMP (5), were added. The contents of the tube were mixed well and incubated at 37° for 10 minutes, at which time the phosphorylase assay was terminated by the addition of TCA. Suitable aliquots were analyzed for inorganic phosphate, and the units of LP formed could be calculated as described previously (6). A more convenient method was to terminate the phosphorylase assay by transferring 0.2 ml. of the mixture to 1.0 ml. of a solution containing 1 mg. of I_2, 2 mg. of KI, and 0.01 m.eq. of HCl. After dilution to 10 ml., the iodine color was read against a water blank in a Klett-Summerson photometer, the No. 54 filter being used.

Materials—The 11,000 × *g* supernatant fraction used in the assay of cyclic 3,5-AMP was prepared either from homogenates of preincubated dog liver slices, as described previously (1), or from Waring blendor homogenates of fresh dog liver by centrifugation of the 2000 × *g* supernatant fluid at 11,000 × *g* for 15 minutes in the Spinco preparative ultracentrifuge. Small aliquots of the 11,000 × *g* supernatant fraction were frozen in liquid N_2 and could be stored either at −20° or −70° for several weeks without obvious deleterious effects.

Dephospho-LP was prepared from dog liver, as described previously (7). Samples of crystalline glucagon, both solid and 0.1 per cent clinical solution, were donated by Eli Lilly and Company. Epinephrine was obtained commercially as a 1 per cent solution. Crystalline disodium ATP was purchased from the Pabst Laboratories. Disodium ATP-8-C^{14} (1 μc. per mg.) was obtained from the Schwarz Laboratories, Inc., and was diluted with an equal weight of Pabst ATP before use. Tris was recrystallized before use (5).

EXPERIMENTAL

Assay of Cyclic 3,5-AMP—The estimation of cyclic 3,5-AMP was based on its ability to stimulate the formation of LP by fractions of liver homogenates. A number of preparations, from the whole homogenate to the

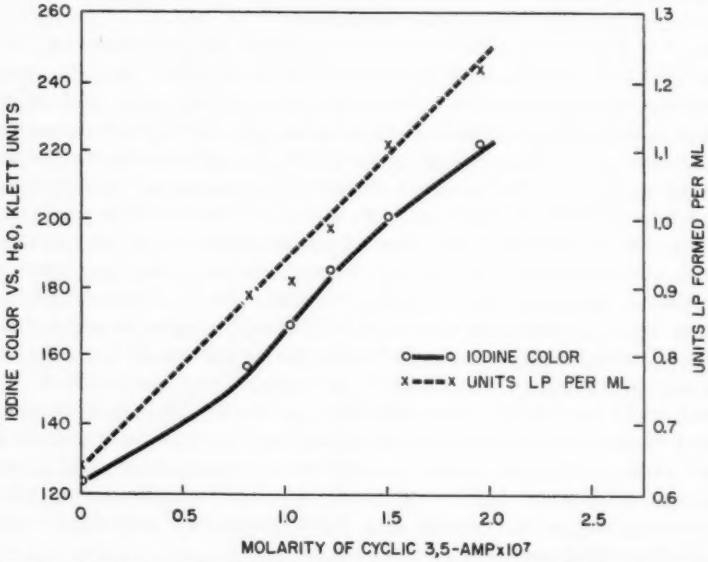

FIG. 1. Standard curves for the assay of cyclic 3,5-AMP. Mixtures containing 3.63×10^{-2} M Tris (pH 7.5), 2.26×10^{-3} M $MgSO_4$, 1.8×10^{-3} M ATP, 9×10^{-4} M caffeine, 4 to 6 units per ml. of dephospho-LP, 0.20 ml. of an 11,000 $\times$ *g* supernatant fraction of a liver homogenate (diluted 20-fold in 0.25 M sucrose), and from 0.0 to 0.6×10^{-4} μmole of pure cyclic 3,5-AMP in a final volume of 0.30 ml. were incubated for 10 minutes at 30°. The LP formed was estimated as described in the text by the addition of a phosphorylase assay reagent, incubation for 10 minutes at 37°, and removal of an aliquot for the determination of inorganic phosphate released from glucose 1-phosphate (upper curve). Another aliquot was placed in a mixture of I_2 and KI, diluted to 10 ml., and the resulting polysaccharide iodine color was read against a water blank in a Klett-Summerson photometer by using the No. 54 filter (lower curve).

dialyzed 100,000 $\times$ *g* supernatant fraction, exhibited graded responses to very low concentrations of the compound. The 11,000 $\times$ *g* supernatant fraction was selected for its ease of storage and relatively low blank rate of LP formation. Fig. 1 shows typical standard curves, in which LP formation is expressed not only in terms of units of phosphorylase activity (5) derived from the measurement of inorganic phosphate released from glucose 1-phosphate, but also in terms of the optical density of the iodine

color of the glycogen formed. The useful titration range was approximately from 0.8×10^{-7} M to 2.0×10^{-7} M cyclic 3,5-AMP. The standard solutions employed were samples purified and characterized as described in Sutherland and Rall (2), and whose concentration was calculated from their ultraviolet absorption by using a millimolar extinction coefficient of 14.2 at 260 mμ.

This assay system proved satisfactory for the estimation of cyclic 3,5-AMP in crude heated extracts from various tissues and in fractions obtained during ion exchange chromatography of heated extracts. Occasionally, heated extracts and chromatographic fractions were encountered which appeared to inhibit the formation of LP in the assay system, giving rise to falsely low values for their cyclic 3,5-AMP content. At present, the substances responsible for this inhibition remain unknown.

The effects on the assay system of some of the known major components of crude heated extracts were evaluated. Since caffeine was included in the assay system in amount sufficient to inhibit maximally enzymatic destruction of cyclic 3,5-AMP (2), there was no effect of adding caffeine in amounts equivalent to that contained in the heated extracts. NaF, at concentrations of 1×10^{-3} M to 1×10^{-2} M, would increase LP formation in the assay system, probably due to its inhibitory action on LP phosphatase (8). However, at 1.7×10^{-3} M, the maximal concentration achieved in the assay of undiluted heated extracts, the effect of NaF was relatively small and could be estimated by suitable controls and subtracted. Additional ATP and $MgSO_4$, in equimolar amounts, increased the formation of LP in the assay system. The addition of ATP alone to a concentration exceeding that of Mg^{++} strongly inhibited LP formation, as noted earlier in the homogenate system used for the assay of glucagon and epinephrine (6). Increasing the ATP and $MgSO_4$ concentrations nearly 5-fold, corresponding to the addition of 2 μmoles of ATP in a final volume of 0.3 ml., increased LP formation about as much as did the addition of 3.0×10^{-5} μmole of cyclic 3,5-AMP. However, the addition of 0.1 μmole of ATP, the most that could be contained in the aliquots of undiluted heated extracts assayed, was without visible effect. Neither 5′-AMP nor a mixture of 2′- and 3′-AMP, tested at final concentrations of 3×10^{-4} to 3×10^{-6} M, had any effect on the assay system either in the presence or the absence of cyclic 3,5-AMP.

The specificity of the assay system has not been extensively surveyed. A number of adenine mononucleotides, including muscle and yeast AMP, adenosine-2′,3′-phosphoric acid, ADP, and moderate amounts of ATP, were inactive. In addition, the di- and trinucleotides, pApA and pApApA,[4]

[4] We thank Dr. Leon Heppel of the National Institutes of Health, Bethesda, Maryland, for kindly supplying samples of these nucleotides.

were inactive when tested at 2.0×10^{-7} M and 1.0×10^{-7} M, respectively. The only substance encountered which mimicked cyclic 3,5-AMP was trypsin. In concentrations of 0.1 to 1.0 γ per ml., the addition of trypsin to the assay system would result in a graded increase in LP formation, while above 1 γ of trypsin per ml., there was strong inhibition of LP accumulation. Both the stimulatory and inhibitory effects on LP formation

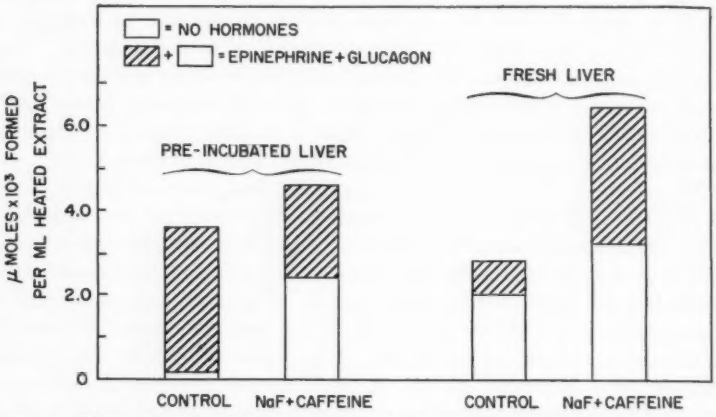

FIG. 2. Effect of epinephrine and glucagon on the formation of cyclic 3,5-AMP. 1.25 ml. aliquots of washed liver particles were incubated for 7 minutes at 30° with 0.04 M Tris (pH 7.5), 2×10^{-3} M ATP, 2.5×10^{-3} M $MgSO_4$, and 1.25 ml. of 0.25 M sucrose in a final volume of 3.0 ml. One-half of the vessels contained 5.5 γ per ml. of epinephrine and 1 γ per ml. of glucagon; in addition, one-half of the vessels contained 0.01 M NaF and 6.67×10^{-3} M caffeine. After incubation, 1 ml. aliquots were heated for 3 minutes in boiling water. The soluble portion of the heated extracts was assayed as described in the text. The total height of the bars represents the cyclic 3,5-AMP formed in the presence of the hormones, and the open portion of the bars represents that formed in their absence. The left-hand pair of bars refers to a particulate preparation derived from preincubated liver slices homogenized in an all-glass homogenizer, and the right-hand bars refer to a particulate preparation derived from a portion of the same liver homogenized in a Waring blendor without previous incubation.

were blocked by crystalline soy bean trypsin inhibitor. This action of trypsin and its relationship to the action of cyclic 3,5-AMP have not been explored further.

Conditions for Formation of Cyclic 3,5-AMP—Earlier experiments had been designed to demonstrate large effects of added epinephrine and glucagon on the formation of cyclic 3,5-AMP (factor) (1). The conditions employed correspond to those in the experiment depicted in the control bar at the left in Fig. 2; *i.e.*, short incubation periods with particles derived from

preincubated liver slices. It became desirable to increase the yield per gm. of liver, as well as the amount of liver processed, in order to accumulate enough material for purification and identification. Particulate preparations obtained from Waring blendor homogenates of fresh tissue were found to be equally active and even exhibited some hormone response

TABLE I

Effect of Various Conditions on Production of Cyclic 3,5-AMP

Heated extracts were prepared by incubating washed particles derived from a Waring blendor homogenate of fresh liver for 30 minutes at 30°. The complete system contained 0.04 M Tris buffer (pH 7.5), 2.5×10^{-3} M $MgSO_4$, 2×10^{-3} M ATP, 6.67×10^{-3} M caffeine, 0.01 M NaF, 5.5 γ per ml. of epinephrine, 1 γ per ml. of crystalline glucagon, 1.5 ml. of 0.25 M sucrose, and 1.5 ml. of washed particle suspension in a final volume of 3.6 ml. An aliquot of the initial $2000 \times g$ precipitate fraction was washed with water instead of 0.25 M sucrose, as described in the text. Also, an aliquot of the particle suspension was frozen in liquid N_2, allowed to warm to −65°, and thawed immediately before use. The soluble portion of the heated extracts was assayed for cyclic 3,5-AMP, as described in the text.

Conditions	Cyclic 3,5-AMP formed per ml. heated extract
	μmoles × 10^3
Complete system	20.6*
No epinephrine or glucagon	18.6*
" caffeine	12.0
" $MgSO_4$	1.0
½$MgSO_4$ (6.25×10^{-4} M)	6.0
No NaF	6.0
" ATP	0.4
½ATP (5×10^{-4} M)	10.0
No ATP + 2×10^{-3} M ADP	16.0
Complete + 2×10^{-2} M glutamate	28.0
Particles diluted 1:3	10.0
Water-washed particles, complete system	20.0
Frozen and thawed particles, complete system	18.0

* Average assay of four heated extracts; the remaining values are the average assay of two heated extracts.

(Fig. 2). The addition of NaF and caffeine increased the formation of cyclic 3,5-AMP (Fig. 2; Table I). Caffeine has been found to inhibit enzymes from various tissues which hydrolyze cyclic 3,5-AMP (2). As would be expected, at 0.01 M, NaF was found to inhibit the release of inorganic phosphate from ATP in such particle preparations.

Table I contains the results of an experiment which summarizes the properties of the liver particle system which forms cyclic 3,5-AMP. The effect of caffeine has already been discussed; Mg^{++} ions are required in

amounts at least in excess of the ATP concentration. NaF could act solely by tending to preserve ATP, considering the rate of ATP hydrolysis observed in its absence and the requirement for high levels of ATP. The concentration of ATP required for the formation of cyclic 3,5-AMP seemed especially high in view of the 1 per cent yield observed; *i.e.*, approximately 0.2 μmole per ml. of cyclic 3,5-AMP was formed from 2 μmoles per ml. of ATP. The stimulatory effect of glutamate probably can be explained on the same basis; *i.e.*, it would tend to maintain the ATP concentration. In other experiments pyruvate and fumarate were about as effective as glutamate. In this connection it was observed that anaerobiosis decreased the formation of cyclic 3,5-AMP, and for this reason the large scale experiments, in which adequate aeration was more difficult, were conducted in an atmosphere of 100 per cent oxygen. Substances like pyruvate and glutamate were not included in the large scale production of cyclic 3,5-AMP in order to simplify possible problems of purification.

TABLE II

Effect of Substitution of ADP for ATP in Formation of Cyclic 3,5-AMP

The heated extracts were prepared by incubating 1.5 ml. of a frozen and thawed washed liver particle suspension with 0.04 M Tris (pH 7.5), 2.5×10^{-3} M $MgSO_4$, 6.67×10^{-3} M caffeine, 0.01 M NaF, 5.5 γ per ml. of epinephrine, 1 γ per ml. of glucagon, and 1.5 ml. of 0.25 M sucrose in a final volume of 3.6 ml. at 30°. Either ATP, ADP, or both ATP and ADP were present as indicated. After 5 and 15 minutes of incubation, 1 ml. aliquots of the mixtures were removed and heated in boiling water. The soluble portion of the heated extracts was assayed as described in the text.

Conditions	Cyclic 3,5-AMP formed per ml. heated extract	
	5 min.	15 min.
	μmoles × 10^5	μmoles × 10^5
2×10^{-3} M ATP	2.8	7.6
1×10^{-3} " " + 1×10^{-3} M ADP	1.2	3.4
No ATP + 2×10^{-3} M ADP	0.8	2.0

In the experiment in Table I, ADP was nearly capable of replacing ATP. However, in other experiments with frozen particulate preparations, the formation of cyclic 3,5-AMP was reduced by nearly 70 per cent when ADP was substituted for ATP, even when very short incubation times were employed (Table II). This would indicate that ADP must be converted to ATP before participating in the reaction rather than the reverse. On the other hand, adenosine tetraphosphate displayed little or no ability to replace ATP in this system.

Dilution of the liver particles resulted in the formation of less cyclic

3,5-AMP, usually not in proportion to the dilution factor. In most experiments, at the concentration of particles used in Table I or lower, the concentration of cyclic 3,5-AMP reached a maximum after 20 to 40 minutes and declined with further incubation. A 30 minute incubation period was adopted for the large scale production experiments.

The ability of particulate preparations to form cyclic 3,5-AMP was not greatly impaired after either hypotonic exposure or freezing (Table I). In other experiments a combination of freezing and washing in water did not abolish the response of liver particulate preparations to epinephrine or glucagon.

TABLE III

Conversion of ATP-8-C^{14} to Radioactive Cyclic 3,5-AMP

The heated extract was prepared by shaking 60 ml. of a suspension of water-washed liver particles at 30° for 20 minutes in a system containing 0.04 M Tris (pH 7.5), 2.5×10^{-3} M $MgSO_4$, 6.67×10^{-3} M caffeine, 0.01 M NaF, 5.5 γ per ml. of epinephrine, 1 γ per ml. of glucagon, and 60 ml. of 0.25 M sucrose in a final volume of 144 ml. The incubation mixture also included 2×10^{-3} M ATP containing 95 μc. of ATP-8-C^{14} (0.5 μc. per mg.) and was flushed with 100 per cent oxygen. 0.90 μmole of cyclic 3,5-AMP was formed, of which 0.40 μmole was recovered as the pure compound.* ADP and AMP were recovered as side fractions from ion exchange chromatography of the heated extract. Radioactivity measurements were made with an end window Geiger counter on dried, 0.10 ml. aliquots of suitable dilutions. The concentration of samples counted was calculated from ultraviolet absorption by using a millimolar extinction coefficient at 260 mμ of 14.2 for ATP, ADP, AMP, and cyclic 3,5-AMP.

Sample	Specific activity
	c.p.m. per μmole
ATP before incubation	17,750
ADP recovered from heated extract	16,000†
AMP " " " "	18,000
Cyclic 3,5-AMP	20,580

* The ion exchange chromatographic procedures for the isolation of cyclic 3,5-AMP are described by Sutherland and Rall (2).

† Not corrected for absorption due to contamination of this sample with NaCl.

Formation of Radioactive Cyclic 3,5-AMP from C^{14}-ATP—The low yield of cyclic 3,5-AMP relative to the high concentration of ATP required to sustain the reaction allowed the possibility that some constituent of the particles was actually the precursor of the compound and that the requirement for ATP could be explained on some other basis. To test this possibility, ATP-8-C^{14} was incubated with liver particles, and the radioactivity of the cyclic 3,5-AMP formed was determined after isolation (Table III). The specific activity of the cyclic 3,5-AMP in counts per minute

per micromole was no lower than that of the original ATP solution. The specific activities of ADP and AMP recovered from the heated extract show that little dilution of the added ATP occurred. No ATP was recovered during ion exchange chromatography of the heated extract.

Formation of Cyclic 3,5-AMP in Non-Hepatic Tissues—Observations on the production of cyclic 3,5-AMP were extended to tissues other than liver. In Table IV are listed the results of an experiment in which particulate preparations from the heart, skeletal muscle, and brain of a dog were incubated with ATP with and without epinephrine. Under the conditions used (no NaF) in the absence of epinephrine, no detectable cyclic 3,5-AMP was formed with skeletal and heart muscle preparations, although

TABLE IV

Formation of Cyclic 3,5-AMP in Extrahepatic Tissues

The heated extracts were prepared by incubating 1 ml. of washed particle suspension from the specified tissue at 30° for 5 minutes in a system containing 0.04 M Tris (pH 7.5), 2.5×10^{-3} M $MgSO_4$, 2×10^{-3} M ATP, 6.67×10^{-3} M caffeine, 5.5 γ per ml. of epinephrine, and 1.0 ml. of 0.25 M sucrose in a final volume of 2.4 ml. The control vessels contained no epinephrine. The soluble portion of the heated extracts was assayed as described in the text.

Tissue	Cyclic 3,5-AMP formed per ml. heated extract	
	Control	With epinephrine
	μmole $\times 10^3$	μmoles $\times 10^3$
Heart muscle	0	1.0
Skeletal muscle	0	2.6
Brain	1.0	1.0

a significant amount was formed in the presence of the hormone. In another experiment with skeletal muscle particles, glucagon was ineffective in stimulating cyclic 3,5-AMP formation. With the use of particulate preparations from brain, conditions have not been devised as yet whereby an effect of added epinephrine on the formation of cyclic 3,5-AMP could be observed. In large scale experiments cyclic 3,5-AMP was isolated from heated extracts derived from the incubation of heart, skeletal muscle, and brain particles with ATP and was characterized as described elsewhere (2).

DISCUSSION

In the presence of a crude liver particulate suspension and C^{14}-labeled ATP, cyclic 3,5-AMP was formed with apparently the same amount of radioactivity per adenine residue as the original ATP. This would indi-

cate that the formation of cyclic 3,5-AMP involves the cyclization of ATP without the obligatory contribution of ribonucleotide residues from endogenous polynucleotides. The crude particulate preparations used in this report were capable of rapidly forming inorganic phosphate from either ATP or inorganic pyrophosphate. Therefore, studies on the fate of the remaining 2 phosphate residues of ATP following the synthesis of cyclic 3,5-AMP have been deferred until enzyme preparations containing less of these interfering activities are available.

The relationship of epinephrine and glucagon to the formation of LP now appears to be an indirect one, and the exact mechanism of action of these hormones in liver must be sought in the factors influencing the accumulation of cyclic 3,5-AMP in tissue preparations. The concentrations of the compound that were achieved in incubation mixtures appeared to be the result of a balance of synthesis and hydrolysis by independent enzyme systems, and the increased concentration observed in the presence of the hormones could be explained either by a stimulatory action on the former or by an inhibitory action on the latter process. This analysis ignores the possibility that the formation of cyclic 3,5-AMP is a freely reversible reaction and that the hormones could act by somehow slowing the back-reaction.

Further information on the mechanism of both synthesis of cyclic 3,5-AMP and hormone action will depend on purification, or at least simplification of the tissue preparations used. Purification and characterization of the cellular particles involved may be an important first step in such an approach. Recent preliminary experiments have shown that rat liver particulate fractions are capable of responding to epinephrine and glucagon by forming significant amounts of cyclic 3,5-AMP, despite the fact that rat liver homogenates were relatively unresponsive to the hormones, as judged by stimulation of LP formation (6). It is hoped that fractionation of this much studied tissue, coupled with appropriate biochemical tests, will allow some characterization of the particulate material which catalyzes this reaction.

The observation that the formation of cyclic 3,5-AMP is catalyzed by preparations from extrahepatic tissues, and that in the case of heart and skeletal muscle its accumulation can be influenced by epinephrine, focuses attention on the physiological and pharmacological action of this compound in these tissues. It is possible that cyclic 3,5-AMP and perhaps related compounds may play a significant role in the regulation of metabolism and function of a number of tissues, particularly those on which sympathomimetic amines have an effect. However, the only biochemical event which is at present known to be influenced by cyclic 3,5-AMP is the phosphorylation of dephosphophosphorylase and, as yet, it is difficult to under-

stand the multitude of physiological effects of epinephrine in the light of this one reaction. In any event, investigation of the formation of cyclic 3,5-AMP in extrahepatic tissues would be worth while, since these tissues might provide enzyme systems more readily fractionated than those from liver.

SUMMARY

1. An assay system suitable for the detection of small amounts of adenosine-3′,5′-phosphoric acid (cyclic 3,5-AMP) in crude and fractionated heated extracts is described. The assay depends upon the stimulation by this compound of liver phosphorylase formation in fractions of liver homogenates.

2. The formation of cyclic 3,5-AMP catalyzed by tissue particulate fractions requires the addition of Mg^{++} ions and adenosine triphosphate (ATP). Adenosine diphosphate is much less effective.

3. The accumulation of cyclic 3,5-AMP is increased by NaF, caffeine, which is known to inhibit enzyme or enzymes hydrolyzing this compound, and by epinephrine and glucagon acting in an unknown fashion.

4. C^{14}-labeled ATP gave rise to cyclic 3,5-AMP with sufficient radioactivity to be derived solely from added ATP.

5. Particulate preparations from heart, skeletal muscle, and brain formed significant amounts of cyclic 3,5-AMP under the conditions used for liver. Epinephrine increased the accumulation of this compound in the preparations from heart and skeletal muscle.

The authors wish to thank Miss Arleen M. Maxwell, Mr. James W. Davis, and Mr. Robert H. Sharpley for technical assistance in these studies.

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FRACTIONATION AND CHARACTERIZATION OF A CYCLIC ADENINE RIBONUCLEOTIDE FORMED BY TISSUE PARTICLES*

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(Received for publication, October 23, 1957)

The isolation of an unusual adenine ribonucleotide was noted in a previous report (1); this compound was formed by particulate fractions of liver homogenates in the presence of adenosine triphosphate, magnesium ions, and epinephrine or glucagon. The adenine ribonucleotide was found to be identical (2) to a product isolated from a barium hydroxide digest of adenosine triphosphate by Cook, Lipkin, and Markham (3).[1] These authors originally termed the compound a cyclic dianhydrodiadenylic acid with phosphate esterification to ribose at positions 3 and 5. However, more recent evidence indicates that the compound is the mononucleotide, adenosine-3′,5′-phosphoric acid.[2] In this report the compound will be termed 3,5-AMP[3] for convenience. The formation of this compound in various tissue preparations has been described (4); the experiments reported in this paper describe the purification and certain properties of this ribonucleotide.

Methods

Materials—A highly purified prostatic phosphatase and a commercial sample of Russell's viper venom were supplied by Dr. Henry Sable; a highly purified spleen phosphodiesterase was supplied by Dr. Leon Heppel.

* This investigation was supported in part by a research grant (No. H-2745) from the National Heart Institute of the United States Public Health Service, and a grant-in-aid from the Lilly Research Laboratories.

[1] Dr. Cook, Dr. Lipkin, and Dr. Markham furnished samples which had been isolated by paper chromatography. The criteria of identity have been summarized in a previous note (2).

[2] In a personal communication, Professor David Lipkin has informed us that a molecular weight determination and other data prove that the compound is adenosine-3′,5′-phosphoric acid. The complete proof of structure of the nucleotide will be presented in a forthcoming publication by D. Lipkin, R. Markham, and W. H. Cook.

[3] The following abbreviations are used: cyclic 3,5-AMP, adenosine-3′,5′-phosphoric acid; 5′-AMP, adenosine 5′-phosphate; 2′-AMP, adenosine 2′-phosphate; 3′-AMP, adenosine 3′-phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Tris, tris(hydroxymethyl)aminomethane; D = optical density.

The intestinal phosphatase was purchased from the Nutritional Biochemicals Corporation and was dialyzed *versus* H_2O before use. Crystalline ribonuclease was obtained from the Worthington Biochemical Corporation. Yeast adenylic acid was purchased from the Schwarz Laboratories, Inc., and ribose 5-phosphate was purchased from the Nutritional Biochemicals Corporation.

Assays—The assays of cyclic 3,5-AMP were performed as described (4). Assay of the cyclic 3,5-AMP-inactivating enzyme (phosphodiesterase) involved incubation of 1×10^{-4} M cyclic 3,5-AMP with the enzyme preparation for 10 minutes at 30° in 0.04 M Tris buffer (pH 7.45) and 2×10^{-3} M $MgCl_2$. After heating in a boiling bath for 2 minutes, the reaction mixture was cooled and diluted suitably for assay of residual cyclic 3,5-AMP.

Ion Exchange Resin Chromatography—Analytical grade resins AG 2-X8 chloride and AG 50-X8 hydrogen of 200 to 400 mesh were purchased from the Bio-Rad Laboratories; these had been processed from Dowex 2-X8 and Dowex 50-X8. The resins were washed twice with 5 volumes of 2 N HCl, then twice with distilled water, 2 N NaOH, distilled water, 2 N HCl, and then with glass-distilled water until chloride-free. Such resins were used for the first two steps; resins for later steps were washed further with 0.1 N NaOH, distilled water, 0.1 N HCl, and glass-distilled water.

The pressure of gravity was utilized in all cases for absorption and elution procedures and averaged about 30 cm. for absorption of cyclic 3,5-AMP on Dowex 2 resins at neutral pH, while an average pressure of 15 cm. of fluid was used for elution from Dowex 2 columns and for all steps with Dowex 50 resins. The temperature of the air-conditioned room varied from about 22–28°.

EXPERIMENTAL

Isolation of Cyclic 3,5-AMP

Preparation of Heated Extracts—Heated extracts containing cyclic 3,5-AMP were prepared by the procedure described previously (4), adapted to a large scale. Washed particles derived from one dog liver, treated in a blendor in 0.25 M sucrose, were incubated in a medium containing at final concentrations 0.04 M Tris (pH 7.5), 2.5×10^{-3} M $MgSO_4$, 2×10^{-3} M ATP, 6.67×10^{-3} M caffeine, 0.01 M NaF, 5.5 γ per ml. of epinephrine, 1 γ per ml. of glucagon, and approximately 0.2 M sucrose. On the average, each liter of incubation mixture contained washed particles derived from about 280 gm. of liver. The mixtures were incubated at 30° for 30 minutes with mechanical stirring in an atmosphere of 100 per cent O_2 and then were heated for 5 to 7 minutes in boiling water. After chilling, the insoluble material was removed by centrifugation and the supernatant fluid (heated or boiled extract) was stored at −20°.

First Dowex 2 Resin Column (1-D-2)—Table I summarizes the results obtained during the fractionation of a pooled sample of boiled extracts derived from four livers. 6.5 liters of boiled extract were passed overnight through a Dowex 2 resin column measuring 15 cm. × 10.5 sq. cm. The resin was then washed with 100 ml. of H_2O and was eluted with 0.05 N HCl (the combined volume of Fractions 1 to 5 was 540 ml., and each subsequent fraction measured 60 ml.). In Fraction 6, the pH of the effluent decreased sharply to produce a blue color with Congo red indicator. This change in pH preceded slightly the elution of cyclic 3,5-AMP and served as a guide in fractionation, since the fraction at which this change occurred varied somewhat, depending upon the volume of heated extract passed through the resin. Assays for cyclic 3,5-AMP showed that the major portion of the compound was contained in Fractions 8 to 13 (Table I), which

TABLE I

Fractionation of Cyclic 3,5-AMP Formed by Liver Particles

The boiled extracts from four liver particle incubation mixtures were pooled and the 6470 ml. were applied to the 1-D-2 resin column and eluted with 0.05 N HCl. Fractions 8 to 13 were pooled and passed through the 1-D-50 resin column, followed by 0.05 N HCl as the continued eluting agent. Fractions 4 to 8 were pooled, neutralized, and, after addition of Tris, were applied to the 2-D-2 resin column and eluted with 0.02 N HCl. Fractions 2 to 5 were pooled and passed through the 2-D-50 resin column, followed by 0.02 N HCl as the continued eluting fluid. The value of optical density per micromole of cyclic 3,5-AMP was calculated to be 14.2 for pure cyclic 3,5-AMP.

Fraction		$D_{260} \times$ ml.	$\frac{D_{230}}{D_{260}}$	$\frac{D_{250}}{D_{260}}$	Cyclic 3,5-AMP*	$\frac{D_{260} \times \text{ml.}}{\mu\text{moles cyclic 3,5-AMP}}$
					μmoles	
Boiled extract		748,000	0.850	0.740	70	10,700
1-D-2	7	5,160	0.356	0.803	1.08	4,780
1-D-2	8–13	30,200	0.332	0.823	54	560
1-D-2	14	6,960	0.392	0.872	5.8	1,200
1-D-50	3	145	0.620	0.926	0.40	362
1-D-50	4–8	1,360	0.829	0.833	46	29.6
1-D-50	9	390	1.47	0.611	0.68	573
2-D-2	1	193	1.90	0.478	0.12	1,610
2-D-2	2–5	584	0.459	0.876	38	15.4
2-D-2	6	16.5	0.490	0.902	0.30	55.0
2-D-50	2	2.9	0.745	0.889	0.068	42.7
2-D-50	3	68	0.238	0.902	5.0	13.6
2-D-50	4	228	0.234	0.877	16.8	13.6
2-D-50	5	189	0.337	0.896	13.2	14.3
2-D-50	6	59	1.04	0.702	2.4	24.5
2-D-50	7	34	1.71	0.528	0.36	94.5

* Calculated from activity in the assay system (4).

also encompassed the last two-thirds of the 5′-AMP peak and the first one-third of the ADP peak. The 1-D-2 column served mainly to concentrate cyclic 3,5-AMP and to remove large amounts of various impurities.

First Dowex 50 Resin Column (1-D-50)—Fractions 8 to 13 from the 1-D-2 column were pooled and passed through a Dowex 50 resin column measuring 15 cm. × 7.0 sq. cm. previously washed with 0.05 N HCl. For continued elution, 0.05 N HCl was added. The combined volume of Fractions 1 and 2 was 450 ml., while each subsequent fraction was 134 ml. Practically all the ADP appeared in Fractions 1 and 2, and almost all the AMP was still on the resin after Fraction 9 was collected. These nucleotides had been present in large excess over the cyclic 3,5-AMP, which, in this case, was confined mainly to Fractions 4 to 8 (Table I). This step also removed a considerable amount of buffering material which appeared to be primarily inorganic phosphate.

Second Dowex 2 Resin Column (2-D-2)—Fractions 4 to 8 from the 1-D-50 column were pooled and neutralized, and, after the addition of 0.2 per cent volume of 1.0 M Tris (pH 7.45), were passed overnight through a Dowex 2 resin column measuring 15 cm. × 0.79 sq. cm. The next morning the resin was washed successively with 10 ml. of H_2O and 50 ml. of 0.005 N HCl; 0.02 N HCl was then added, and 10 ml. fractions were collected. In general, this step separated almost all other components from cyclic 3,5-AMP, at least in selected fractions. However, some difficulty was encountered in freeing the Dowex 2 resin of elutable ultraviolet-absorbing material which then contaminated presumably all fractions. Fortunately, this material appeared to be retained on Dowex 50 resins at acid pH. In addition, a number of the fractions containing a considerable portion of the cyclic 3,5-AMP frequently contained small amounts of other nucleotides, as was obviously the case in the example shown in Table I.

Second Dowex 50 Resin Column (2-D-50)—Fractions 2 to 5 from the 2-D-2 column were pooled and passed through a Dowex 50 resin column measuring 15 cm. × 1.13 sq. cm. previously washed with 0.02 N HCl; for continued elution, 0.02 N HCl was added. The volume of Fraction 1 was 40 ml., and each subsequent fraction was 20 ml. The impurities removed by this step appear to be compounds containing cytosine and guanine, as judged by the ultraviolet spectrum (Table I). The theoretical limit of 14.2 for D_{260} × ml. per micromole (millimolar extinction coefficient) was occasionally exceeded slightly, as shown in Table I, last column. Whenever highly active fractions have been reassayed and refractionated, all results have indicated that a value of about 14.2 represents maximal activity. The over-all recovery of cyclic 3,5-AMP in Fractions 3, 4, and 5 from the 2-D-50 column was 50 per cent.

Crystallization of Cyclic 3,5-AMP—Several preparations from the 2-D-2

fractionation, similar to Fractions 2 to 5 in Table I, were pooled, neutralized, and fractionated again on a Dowex 2 chloride resin column. The most active fractions were pooled and fractionated further on a Dowex 50 resin column, and the peak fractions yielded 27 ml. of a 1.2×10^{-3} M solution in 0.05 N HCl with high activity (D_{260} per micromole = theory). This sample (Special D-50) was lyophilized without neutralization. To one-half of the resultant powder in a small flask was added 1.0 ml. of glass-distilled water, and the flask and contents were warmed to 50°. The sample was transferred to a centrifuge tube and, on chilling, crystals appeared with a pronounced sheen. In general, small rods often in rosettes were most common; the ends were generally pointed when the rods were larger. At times, flat rods or plates with pointed tips were formed; occasionally these crystals were large and readily visible with 100×, or less magnification. The warmed solution was 3.6×10^{-2} M with a pH of about 2, while the supernatant fluid above the crystals at 2° was 0.9×10^{-2} M.

Isolation of Cyclic 3,5-AMP from Extrahepatic Tissues—Boiled extracts were prepared from incubation mixtures in which particles from dog heart, skeletal muscle, and brain were substituted for liver particles (4). These boiled extracts were fractionated by ion exchange resins as described above, except that smaller volumes of extracts were used and the resin columns were proportionately of smaller diameter. The results of fractionation on the 2-D-2 resin column are summarized in Table II. Over-all recoveries in Fractions 1 to 4 were 53 per cent for heart and skeletal muscle and 45 per cent for brain. It may be noted that the values $D_{260} \times$ ml. per micromole of cyclic 3,5-AMP for Fractions 2, 3, and 4 do not differ greatly from the value obtained with the fractionation described in Table I. Samples 2, 3, and 4 were pooled, passed through Dowex 50, and appropriate fractions were lyophilized and characterized further as reported below.

Properties of Cyclic 3,5-AMP

Spectrum and Components—The ultraviolet spectrum of highly purified samples was similar to that of 5′-AMP. The E_{max} in 0.05 N HCl was at 257 mμ with essentially maximal absorption at 256 mμ. In acid, the $D_{230/260}$ was 0.23 to 0.24, and the $D_{250/260}$ was 0.88 to 0.89; the corresponding values for 5′-AMP were smaller. The spectrum in alkali was almost identical to the spectrum of 5′-AMP.

The assumption was made that the molar extinction coefficient for cyclic 3,5-AMP in acid at 260 mμ was 14.2×10^3. The validity of this assumption was established by experiments reported below in which cyclic 3,5-AMP was converted quantitatively to 5′-AMP without detectable change in optical density at 260 mμ. Ribose content was determined (5), and

phosphate content (6) after ashing. Based on the above assumption of extinction, it was found that 1.00 μmole of cyclic 3,5-AMP contained 1.01 μmoles of ribose and 1.02 μmoles of phosphate. Several tests for other components were negative and included ninhydrin (7) before and after hydrolysis, carbazole (8), carbazole and cysteine (9), naphthoresorcinol (10), and sulfuric acid-cysteine tests (11).

Titration with Alkali—A sample of lyophilized cyclic 3,5-AMP (lyophilized special Dowex 50 preparation) was dissolved in H_2O and 4.4 ml. of

TABLE II

Second Dowex 2 Fractionation of Cyclic 3,5-AMP Formed by Particles from Heart, Skeletal Muscle, or Brain

Fractions 4 to 8 from the 1-D-50 resin column were pooled, neutralized, and fractionated on the 2-D-2 resin column as described in Table I, except that the column size was 15 cm. × 0.125 sq. cm. and the individual fractions were correspondingly smaller.

Tissue	Fraction No.	$D_{260} \times$ ml.	$\frac{D_{230}}{D_{260}}$	$\frac{D_{250}}{D_{260}}$	Cyclic 3,5-AMP	$\frac{D_{260} \times \text{ml.}}{\mu\text{moles cyclic 3,5-AMP}}$
					μmole	
Heart	1	0.260	0.36	0.86	0.010	26
"	2	0.96	0.26	0.90	0.040	24
"	3	0.54	0.27	0.93	0.042	13
"	4	0.27	0.30	0.92	0.017	16
Skeletal muscle	1	0.28	0.38	0.90	0.0066	43
" "	2	1.6	0.27	0.89	0.098	16
" "	3	1.1	0.25	0.90	0.064	17
" "	4	0.39	0.40	1.27	0.032	12*
Brain	1	2.4	0.20	0.84	0.005	480
"	2	4.2	0.23	0.89	0.20	21
"	3	2.9	0.23	0.90	0.17	17
"	4	0.98	0.23	0.98	0.060	16

* The corresponding value for Tube 5 was 26.

a 5.8×10^{-3} M solution (pH 2.30) were titrated with 0.10 N NaOH to neutrality. A HCl solution at pH 2.35 served as a control. It was found that more alkali was required to neutralize the cyclic 3,5-AMP solution than the control. Buffering was found to occur in the area near pH 4.0, but essentially no buffering was noted in the pH range 5.5 to 7.0. This indicated that no monoesterified phosphate groups were present.

Stability in Acid and Alkali—The stability of cyclic 3,5-AMP in acid was determined by measurement of biological activity and by formation of inorganic phosphate. Cyclic 3,5-AMP in 1.0 N HCl was heated in boiling water and samples were removed at 15, 30, and 60 minutes. At these

times, the losses of biological activity were 19, 54, and 78 per cent, respectively, and the amounts of inorganic phosphate formed were 17, 41, and 63 per cent of the total. Cyclic 3,5-AMP in 1.0 N NaOH was also heated in boiling water and samples were removed at 7, 15, and 40 minutes. At these times, the losses of biological activity were 31, 64, and 90 per cent. These and other experiments showed that the nucleotide was very resistant to inactivation by acid or alkali.

Hydrolysis with Dowex 50 and Identification of Products

The hydrogen form of Dowex 50 has been used as a catalyst for hydrolysis of the glycosidic link of adenine ribonucleotides. With use of this catalyst, the hydrolysis was so rapid that ribose 2-phosphate and ribose 3-phosphate were formed from 2′-AMP and 3′-AMP, respectively, without significant isomerization (12). However, in preliminary experiments cyclic 3,5-AMP was hydrolyzed only about 35 per cent after 15 minutes heating in the presence of Dowex 50 and 0.05 N HCl. Under these conditions, isomerization of phosphate attached to the 2 or 3 position of ribose might be expected. In these experiments the ribose phosphate or phosphates formed from cyclic 3,5-AMP differed from ribose 5-phosphate in having a slower rate of color development with orcinol reagent, and a faster rate with the sulfuric acid-cysteine reagents. The ribose phosphate was more acid-labile than ribose 5-phosphate and was eluted from Dowex 2 by NH_4Cl in the presence of borate.

A larger scale experiment was performed to produce amounts of ribose phosphate sufficient for characterization by chromatography. A Dowex 50 resin sample was washed three times with 0.05 N HCl and upon centrifugation at 1200 × *g* packed to a volume of 2.9 ml. To this were added 10 ml. of cyclic 3,5-AMP in 0.05 N HCl containing the equivalent of 600 γ of ribose. The mixture was stirred for 20 minutes at room temperature, then heated in a boiling bath for 25 minutes with stirring, cooled to room temperature, and centrifuged for 7 minutes at 1200 × *g*. The supernatant fluid was removed, and to the precipitate were added 10 ml. of 0.05 N HCl. The suspension was heated in the boiling water bath for 25 minutes with stirring, cooled, and, after centrifugation, the supernatant fluid was removed and pooled with the previous supernatant fluid. The pooled supernatant fluids contained ribose or ribose derivatives equivalent to 490 γ of ribose. After neutralization and addition of 2 per cent by volume of 0.2 M Tris, pH 7.4, the sample was passed over a Dowex 2 chloride resin column 12.2 cm. × 0.28 sq. cm. The resin was washed with H_2O and eluted with 0.015 N HCl. (Preliminary experiments showed that ribose phosphates were separated from cyclic 3,5-AMP and ribose by this fractionation.) The eluted fractions contained 93 per cent of the applied ribose or ribose

derivatives and it was estimated that 72 per cent of the eluted material was present as ribose phosphate, 14 per cent as free ribose, and about 14 per cent as nucleotide.

The ribose phosphates were pooled and the major portion was fractionated on an anion exchange resin as described in Fig. 1, a modification of the methods of Khym and Cohn being used (12, 13). A mixture of ribose 3-phosphate and ribose 2-phosphate was prepared from yeast adenylic

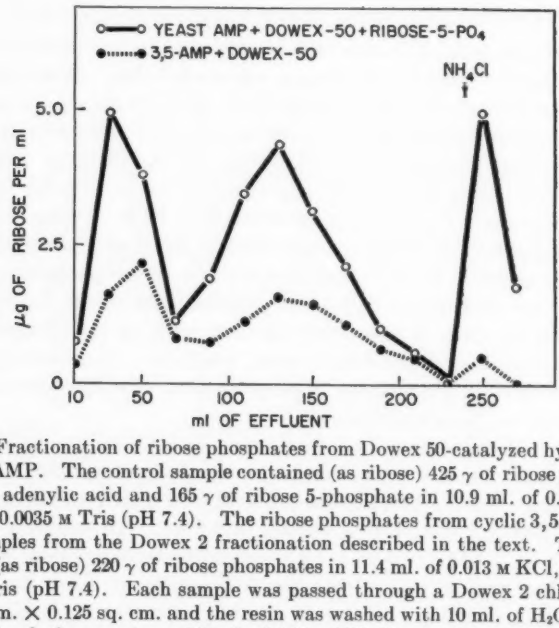

FIG. 1. Fractionation of ribose phosphates from Dowex 50-catalyzed hydrolysis of cyclic 3,5-AMP. The control sample contained (as ribose) 425 γ of ribose phosphates from yeast adenylic acid and 165 γ of ribose 5-phosphate in 10.9 ml. of 0.013 M KCl, containing 0.0035 M Tris (pH 7.4). The ribose phosphates from cyclic 3,5-AMP were pooled samples from the Dowex 2 fractionation described in the text. The sample contained (as ribose) 220 γ of ribose phosphates in 11.4 ml. of 0.013 M KCl, containing 0.0035 M Tris (pH 7.4). Each sample was passed through a Dowex 2 chloride resin column 5 cm. $\times$ 0.125 sq. cm. and the resin was washed with 10 ml. of H_2O, and then eluted. The eluting agent was 240 ml. of 0.04 M NH_4Cl, containing 0.002 M $Na_2B_4O_7$; then 0.04 M NH_4Cl alone was added, as indicated by arrow.

acid in a similar fashion and was fractionated by the same methods. Pilot experiments showed that the ribose phosphates were separated by relatively short resin columns. The results of the experiment summarized in Fig. 1 indicated that the ribose phosphates formed from cyclic 3,5-AMP in the presence of the hydrogen form of Dowex 50 were ribose 3-phosphate and ribose 2-phosphate. Only about 5 per cent of the total ribose was found in the fractions in which ribose 5-phosphate was eluted after addition of NH_4Cl without borate. All of the ribose content applied to the columns was accounted for in the eluted fractions shown in Fig. 1.

The Dowex 50 resin present after hydrolysis of the cyclic 3,5-AMP was washed with 0.05 N HCl and then eluted. To a control resin were added 3 μmoles of adenine in 0.05 N HCl, and after stirring for 30 minutes at room temperature, the control resin was treated exactly as the experimental resin. The resins were placed in glass columns forming a bed 3.6 × 0.79 sq. cm. and, after washing with 70 ml. of 0.05 N HCl, to each was added 0.5 N KOH until the pH of the effluent became suddenly alkaline, at which time 0.05 N KOH was used to elute the resins. From these alkaline fractions were recovered, on the basis of spectrum, 3.1 μmoles of adenine from the control and 2.3 μmoles from the resin incubated with cyclic 3,5-AMP. The peak fractions were passed over Dowex 2 and the spectra of fractions were identical in acid and alkali. The absorption maximum of both samples was 263 mμ in 0.1 N HCl and 269 mμ in 0.1 N KOH.

Enzymatic Hydrolysis of Cyclic 3,5-AMP and Identification of Product

Preparation of Enzymes—Fresh beef hearts were chilled and the ventricles were ground in a meat grinder. The ground muscle was homogenized with 2 volumes of cold 0.33 M sucrose in a Waring blendor for about 1 minute until the tissue was well dispersed. The homogenate was centrifuged at 4100 × *g* for 15 minutes and the supernatant fluid (sucrose extract) was decanted.

Solid ammonium sulfate was added to the sucrose extract in amount sufficient to produce a 45 per cent saturated solution (31.5 gm. of ammonium sulfate plus 1.37 ml. of 1 N KOH per 100 ml.). After standing for 20 minutes in an ice bath, the precipitate was collected by centrifugation at 7000 × *g* for 15 minutes, and the supernatant fluid was discarded. The precipitate was suspended or dissolved with glass-distilled water, the volume added being 10 per cent of the volume of the sucrose extract. A neutralized solution of ammonium sulfate (pretreated with Versene (14) and saturated at room temperature) was added in volume equal to the previous water addition. After standing for 10 minutes in an ice bath, the precipitate was collected by centrifugation at 7000 × *g* for 15 minutes, and the supernatant fluid was discarded. The precipitate (0.5 precipitate) was suspended or dissolved in glass-distilled water, the volume added being 4 per cent that of the sucrose extract, and the preparation was then frozen at −20° for overnight or longer.

The 0.5 precipitate was thawed, mixed well, and, after centrifugation at 10,000 × *g* for 15 minutes, the precipitate was discarded. The supernatant fluid was then centrifuged at 70,000 × *g* for 40 minutes, and the resulting supernatant fluid was decanted and frozen at −20° or fractionated further without freezing. The ammonium sulfate concentration was estimated, and additional saturated ammonium sulfate solution was added

to produce a final concentration of 50 per cent saturation. After standing 10 minutes in an ice bath, the precipitate was collected by centrifugation at 10,000 × *g* for 15 minutes, was dissolved in glass-distilled water (the volume being about 1.5 per cent of the sucrose extract), and the preparation was dialyzed against 8×10^{-4} N KOH for 2 hours, 4×10^{-4} N KOH for 1 hour, and glass-distilled water for 20 hours in the cold. The dialyzed preparation was diluted 10-fold with cold glass-distilled water and 0.1 volume of calcium phosphate gel (15) was added. After 20 minutes of stirring, the gel was collected and washed once with water and twice with 0.1 M Tris (pH 7.45). The gel was dissolved by the addition of 0.1 M citrate, pH 6.5 (4 per cent of sucrose extract volume). This hazy solution was dialyzed for 2 hours *versus* 0.05 M citrate, pH 6.5, then the precipitate, collected by centrifugation at 10,000 × *g* for 15 minutes, was discarded. Ammonium sulfate was added to the supernatant fluid to 14 per cent saturation and the resulting precipitate was again discarded. At times, formation of this precipitate was aided by freezing. The 14 to 40 per cent saturated ammonium sulfate fraction was collected, was dissolved in water (0.5 per cent of sucrose extract volume), and was dialyzed *versus* dilute KOH and water as above. The slightly hazy solution was clarified by centrifugation at 100,000 × *g* and the resulting supernatant fluid served as the partially purified enzyme from heart. This enzyme was activated by magnesium ions.

Extracts from brain were more active than extracts from heart and were fractionated by a similar procedure; however, traces of interfering enzyme, or enzymes, remained in the final brain preparation. Extracts from liver had relatively less activity and have not been fractionated. The enzymatic activity of extracts of heart, brain, and liver was inhibited by caffeine.

Hydrolysis and Identification of Products—The cyclic 3,5-AMP was not inactivated when incubated with prostatic phosphatase, crude intestinal phosphatase, Russell's viper venom (1), nor by incubation with ribonuclease or highly purified spleen phosphodiesterase. However, as reported (1), cyclic 3,5-AMP was rapidly inactivated by extracts from brain and heart, and less rapidly by extracts from liver. In the following experiments the partially purified enzyme from heart was used and was found to release no inorganic phosphate when incubated with 5′-AMP, 3′-AMP, or 2′-AMP, and contained no adenosinetriphosphatase activity. In one experiment the enzyme was incubated with ribonucleic acid core (16) and did not attack this substrate under the conditions employed.

Preliminary experiments showed that the enzymatic inactivation of cyclic 3,5-AMP, catalyzed by the heart enzyme, proceeded without formation of inorganic phosphate and with spectral change limited to a slight lowering of the $D_{280/260}$ and $D_{250/260}$ ratios. Experiments such as the one summarized in Table III established that cyclic 3,5-AMP was quantitatively

converted to 5′-AMP by the heart enzyme as judged by enzymatic dephosphorylation of the product by the 5′-nucleotidase contained in Russell's viper venom and by paper chromatography of the reaction mixture. Since the reaction mixtures were not deproteinized before application to the paper, 5′-AMP was also incubated with the heart enzyme to serve as a

TABLE III

Characterization of Product Formed on Enzymatic Inactivation of Cyclic 3,5-AMP

The reaction mixture (3.6 ml.) contained 4.8×10^{-4} M cyclic 3,5-AMP, 2×10^{-3} M $MgSO_4$, 4×10^{-2} M Tris (pH 7.45), and an amount of partially purified heart enzyme capable of inactivating the cyclic 3,5-AMP completely in less than 60 minutes at 30°. A control reaction mixture contained 4.6×10^{-4} M 5′-AMP. At zero time and after 60 minutes of incubation at 30°, samples were removed for determination of activity, ultraviolet spectrum, migration on paper, and for incubation with several phosphatases. Chromatography was carried out at 22° for 17 hours with No. 1 paper. Prostatic phosphatase was incubated at pH 5.6, and Russell's viper venom at pH 7.4; an excess of enzyme was used in both cases.

Compound	Paper chromatography		Inorganic phosphate released per μmole compound by	
	Solvent 1*	Solvent 2†	Russell's viper venom	Prostatic phosphatase
	R_F	R_F	*μmoles*	*μmoles*
2′-AMP	0.37	0.29	0.0	1.15
3′-AMP	0.22	0.28	0.0	1.0
5′-AMP	0.42	0.26	0.98	1.05
Cyclic 3,5-AMP	0.15	0.33	0.0	0.0
" " + heart enzyme	0.44	0.25	1.09	0.97
5′-AMP + heart enzyme	0.44	0.25	1.05	1.02

* $(NH_4)_2SO_4$, isopropanol, acetate (pH 6.0); ascending (17).

† 95 per cent ethanol, ammonium citrate (pH 4.4); descending (18).

control. Small amounts of protein were sometimes observed to alter the rate of migration, especially in the ascending system used.

Cyclic 3,5-AMP from Heart, Brain, and Skeletal Muscle

Samples from 2-D-50 columns were lyophilized to obtain sufficient concentration for comparison with cyclic 3,5-AMP from liver particles. Samples obtained from heart, brain, and skeletal muscle sources were identical to cyclic 3,5-AMP from liver by the following criteria: (*a*) ultraviolet spectrum, (*b*) biological activity, (*c*) paper chromatography, (*d*) loss of biological activity when incubated with partially purified enzyme from heart, and (*e*) quantitative conversion to 5′-AMP on incubation with the partially purified enzyme from heart.

Cyclic 3,5-AMP from $Ba(OH)_2$ Digest of ATP

Cook, Lipkin, and Markham have isolated a cyclic adenylic acid which was produced during the barium hydroxide hydrolysis of ATP (3). By a number of criteria this compound was found to be identical to the adenine ribonucleotide (cyclic 3,5-AMP) produced in the presence of cellular particles (2). Additional supporting evidence for identity includes the fractionation of the synthetic compound on Dowex 50 resin columns, and subsequent crystallization of the compound. The cyclic nucleotide was synthesized by the method of Cook, Lipkin, and Markham by incubating 5.0 gm. of ATP in 60 ml. of saturated $Ba(OH)_2$ solution in a boiling bath for 35 minutes with shaking. 5 N H_2SO_4 was added in amounts sufficient to precipitate all barium and to bring the pH to about 2. The supernatant fluid was passed through a Dowex 50 column 15 cm. × 7.1 sq. cm. and 0.05 N HCl was added as in the first Dowex 50 step in Table I. By this procedure the cyclic 3,5-AMP was separated from the other adenine nucleotides. The fractions containing cyclic 3,5-AMP were lyophilized, and a concentrated solution was prepared by adding 10 ml. of H_2O to one-half of the powder, and warming to 42°. After brief centrifugation, the supernatant fluid was chilled in a centrifuge tube and crystals appeared as the temperature approached 30°. After chilling to 2°, the crystals were separated by centrifugation, dissolved in H_2O at 42°, and recrystallized by chilling. When the resultant crystals were obtained by centrifugation and diluted to a 3.8×10^{-2} M solution, chilling in a cold room or ice bath was required for crystal production. At 2°, the supernatant fluid above the crystals contained cyclic 3,5-AMP at a concentration of 0.90×10^{-2} M.

In later experiments it was found that the disodium salt was much more soluble in water than the free acid form. Lyophilized cyclic 3,5-AMP could be readily dissolved in dilute NaOH at room temperature to make a solution at pH 5.0 which was several times as concentrated as that achieved when water at 42° was used. Upon acidification with HCl, crystals were obtained; recrystallization from water was performed as before. With this procedure, 490 mg. of crystals were obtained from 15 gm. of disodium ATP, which is about 6 per cent of the theoretical yield.

DISCUSSION

The adenine ribonucleotide isolated from animal tissues has been shown to be identical (2) with a product of the alkaline degradation of ATP isolated by Cook, Lipkin, and Markham (3). The pooled information on the properties of the compound isolated from both sources is compatible with the formulation of the compound as adenosine-3′,5′-phosphoric acid. This structure accounts for the value of 1 for the molar ratio of adenine to ribose to phosphate as well as for the absence of monoesterified phos-

phate. That the compound contains only diesterified phosphate is supported by a large body of data, including titration with alkali (2), resistance to attack by phosphomonoesterases (1–3), and electrophoretic mobility on paper (3), and is suggested by its behavior during paper and ion exchange chromatography. The sole product of hydrolysis catalyzed by the partially purified enzyme from heart was 5′-AMP, and acid hydrolysis, with Dowex 50 as catalyst, led to the formation of an equilibrium mixture of ribose 2-phosphate and ribose 3-phosphate, indicating the existence of 3′,5′- (or 2′,5′-) phosphodiester bonds.[2]

Cyclic 3,5-AMP was not attacked by a number of phosphodiesterases, including crude intestinal phosphatase, spleen phosphodiesterase, and pancreatic ribonuclease (1), and was hydrolyzed only very slowly by high concentrations of crude *Crotalus adamanteus* venom (2, 3). However, it was apparent from early experiments on the biological production of cyclic 3,5-AMP that, once formed, the compound was subject to rapid destruction while in contact with tissue preparations. Various tissue extracts were fractionated in a search for an enzyme inactivating the compound to give products which would yield information regarding structure. It was possible to purify an enzyme from extracts of heart muscle which quantitatively converted cyclic 3,5-AMP to 5′-AMP. Although extracts of brain were more active in destroying cyclic 3,5-AMP than were extracts of heart muscle, purified brain preparations hydrolyzed the compound to give dephosphorylated and deaminated derivatives of 5′-AMP as well as 5′-AMP itself. Although neither one of these partially purified enzymes has been adequately characterized, it seems probable that they are phosphodiesterases. Extracts of liver were much less active in destroying cyclic 3,5-AMP than those of either heart or brain; however, there was enough activity in preparations of liver tissue to require addition of caffeine for maximal accumulation of the compound (4). The distribution and properties of enzymes inactivating cyclic 3,5-AMP have not been studied in detail; it is possible that a given tissue may contain more than one enzyme catalyzing the formation of 5′-AMP, or perhaps other products, from cyclic 3,5-AMP. Future studies on the mechanism of action of epinephrine and glucagon, as well as of other chemical agents (*e.g.* caffeine), will include consideration of the possible participation of these enzymes. Also, it would be expected that the pharmacological effects of exogenous cyclic 3,5-AMP on various tissues would be influenced, not only by the rate of entry of the compound, but also by its enzymatic destruction.[4]

[4] Rabbit liver slices were found to have increased phosphorylase concentration and decreased glycogen content, accompanied by increased glucose output when incubated in the presence of 2×10^{-5} M cyclic 3,5-AMP. (Smith, L., Reuter, S., Sutherland, E., and Rall, T., unpublished observations.)

SUMMARY

1. An adenine ribonucleotide (formed by particulate fractions of liver homogenates in the presence of adenosine triphosphate, magnesium ions, and epinephrine or glucagon) was isolated in good yield by use of ion exchange resins and was crystallized.

2. An adenine ribonucleotide, produced in the presence of particulate fractions from heart, skeletal muscle, and brain was isolated and found to be identical to the one formed by particulate fractions from liver.

3. The adenine ribonucleotide contained no monoesterified phosphate groups and was quantitatively converted to adenosine 5′-phosphate when incubated with a partially purified enzyme from heart. When hydrolysis of the ribonucleotide was catalyzed by the hydrogen form of Dowex 50, the products were identified as adenine and a mixture of ribose 3-phosphate and ribose 2-phosphate. The evidence indicated that the compound was a cyclic adenylic acid.

4. The cyclic adenylic acid was found to be identical to the cyclic adenylic acid isolated by Cook, Lipkin, and Markham from barium hydroxide digests of adenosine triphosphate and recently determined by these authors to be adenosine-3′,5′-phosphoric acid (cyclic 3,5-AMP).

5. An enzyme capable of inactivating cyclic 3,5-AMP was found in several tissues. The enzyme, probably a phosphodiesterase, was especially active in brain extracts and was partially purified from extracts of brain and heart. The enzyme was activated by magnesium ions and was inhibited by caffeine.

The authors wish to thank Mr. James W. Davis, Miss Arleen M. Maxwell, and Mr. Robert H. Sharpley for technical assistance in these studies.

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THIOOXIDASE, A NEW SULFHYDRYL-OXIDIZING ENZYME FROM PIRICULARIA ORYZAE AND POLYPORUS VERSICOLOR

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(Received for publication, December 6, 1957)

In the course of a study of the enzymes of *Piricularia oryzae* Cav., the fungus causing the blast disease of rice, it was observed that activity of crude polyphenoloxidase preparations from culture filtrates was not inhibited by sodium diethyldithiocarbamate (DEDTC), a widely used inhibitor of copper-containing enzymes. Further investigation revealed that such preparations contained an enzyme which catalyzes the oxidation of DEDTC and related compounds. This enzymatic reaction appears not to have been described in the literature, and the enzyme seems to be distinct from other enzymes operating on sulfhydryl-containing substrates. The enzyme, which was later demonstrated also in culture filtrates from the wood-rotting fungus *Polyporus versicolor*, is referred to in this paper as thiooxidase.

Methods

Cell-free culture filtrates of *P. oryzae* (strain No. 775) and of *P. versicolor*, prepared as described elsewhere (1), were used as the source of the enzyme.

Activity was assayed manometrically in a Warburg apparatus at 30.4°. In a typical experiment, 0.5 ml. of 0.1 M DEDTC was placed in the side arm, 0.1 ml. each of di-*n*-hexylamine and 10 per cent potassium hydroxide together with a filter paper wick was placed in the center well, and, in the main compartment, 1.0 ml. of buffer (McIlvaine, pH 6.0 or 7.0), together with an enzyme solution and water sufficient to make a total volume of 3.0 ml.

Because DEDTC tends to decompose into carbon disulfide and diethylamine at even slightly acid pH, it was necessary to provide a trapping reagent for the volatile carbon disulfide produced. The alkaline di-*n*-hexylamine solution proved adequate at pH near 7, but in some experiments at pH 6 the evolution of carbon disulfide was so rapid that positive pressures resulted. To correct for this, blanks containing all the components except enzyme were always included.

Sodium dimethyldithiocarbamate was prepared from dimethylamine hydrochloride, carbon disulfide, and sodium hydroxide. Potassium dithio-

acetate was synthesized by treating methyl magnesium iodide with carbon disulfide, followed by acidification, steam distillation, and addition of potassium hydroxide. Potassium methyl-, ethyl-, propyl-, isopropyl-, butyl-, and amylxanthates were prepared by treating solutions of the corresponding alcohols with potassium hydroxide and carbon disulfide. All other reagents were obtained from commercial sources and were used without further purification.

Results

Identification of Product of DEDTC Oxidation—The most likely oxidation product of DEDTC appeared to be tetraethylthiuram disulfide (TETDS), according to the following reaction:

$$2(C_2H_5)_2N{-}\overset{\overset{S}{\|}}{C}{-}SH + \tfrac{1}{2}O_2 \rightarrow (C_2H_5)_2N{-}\overset{\overset{S}{\|}}{C}{-}S{-}S{-}\overset{\overset{S}{\|}}{C}{-}N(C_2H_5)_2 + H_2O$$

The oxidation product, which has very low solubility in water, soon causes the reaction mixture to become turbid and later separates in crystalline form. After several hours, the crystalline yellow precipitate was separated by centrifugation and washed thoroughly with water. The product was recrystallized three times from 95 per cent ethanol. The infrared spectra of the purified material and of a similarly recrystallized authentic specimen of TETDS were identical, as were the melting points and mixed melting point (71–72° uncorrected; literature value, 70°).

The oxidation product of sodium dimethyldithiocarbamate was shown in the same way to be tetramethylthiuram disulfide.

There was no evidence of formation of thiuram disulfides from the dithiocarbamates in the absence of enzyme or in the presence of boiled enzyme.

The product from the enzymatic oxidation of thiophenol was obtained as white needles which, after recrystallization from aqueous ethanol, melted at 59.5° (uncorrected). According to the literature, diphenyl disulfide is a white compound, m.p. 61°.

Kinetics and Stoichiometry—As shown in Fig. 1, the rate of enzymatic DEDTC oxidation remains constant so long as the substrate concentration is non-limiting. In Fig. 2 is seen the direct proportionality between the rate of DEDTC oxidation and enzyme concentration and in Fig. 3 the effect of substrate concentration on the rate of the reaction is shown.

According to the equation above, 0.25 mole of oxygen is consumed per mole of substrate oxidized. Experimentally, only 70 to 80 per cent of the theoretical oxygen absorption was observed (Fig. 1), owing, presumably, to the concurrent non-enzymatic non-oxidative decomposition of the substrate. Hydrogen peroxide is not produced during the oxidation of DEDTC, as evidenced by the fact that added catalase did not influence

the oxygen consumption in the presence of enzyme preparations demonstrated to be catalase-free.

Properties of Thiooxidase—The crude enzyme is quite stable at low temperature and may be stored for weeks at 4° without appreciable loss

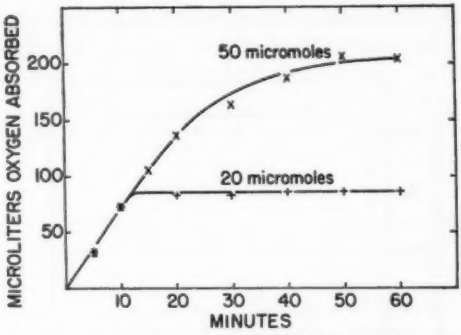

FIG. 1. Rate of enzymatic oxidation of diethyldithiocarbamate as a function of time; pH 6.0. The flask contents were as described in the text. All measurements were corrected for non-enzymatic evolution of carbon disulfide.

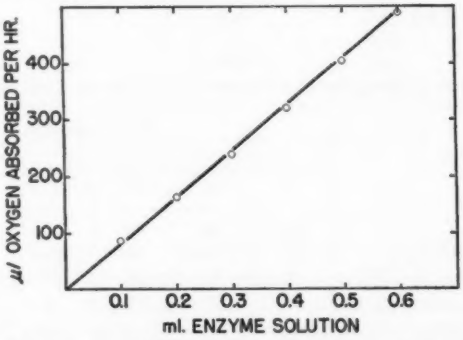

FIG. 2. Rate of oxidation of diethyldithiocarbamate as a function of enzyme concentration; pH 6.0.

of activity. It is inactivated completely after 5 minutes at 100°. 50 per cent acetone produces a precipitate containing most of the activity.

Exhaustive dialysis against distilled water did not diminish activity of the preparations, indicating that no soluble cofactors are involved in the reaction.

Because of the increasingly rapid non-enzymatic decomposition of DEDTC with increasing acidity, only fragmentary data were obtained as

to the pH dependence of the enzymatic reaction. The rate of the enzyme-catalyzed oxidation was considerably less at pH 7.0 than at pH 6.0 and too slow to be measured at pH 8.0.

Activity is inhibited by azide, cyanide, glutathione, and cysteine, as shown in Table I.

Occurrence of Thiooxidase—The enzyme has been demonstrated in the cell-free filtrates from cultures of the rice-blast fungus, *P. oryzae*, and of

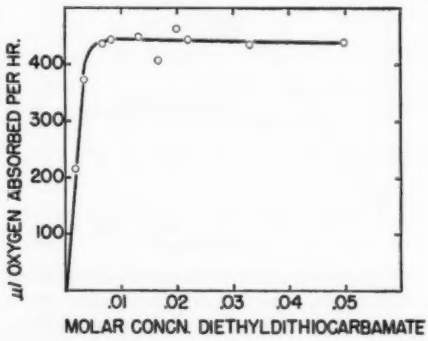

FIG. 3. Rate of oxidation of diethyldithiocarbamate as a function of substrate concentration; pH 7.0.

TABLE I

Influence of Several Heavy Metal Inhibitors on Activity of Thiooxidase at pH 7.0

Inhibitor	Per cent inhibition					
	1.7×10^{-3} M	1.0×10^{-3} M	3.3×10^{-3} M	1.7×10^{-3} M	1.0×10^{-3} M	3.3×10^{-4} M
Azide	100	95	87	75	66	42
Cyanide	96	80	56	38	11	0
Cysteine	90	82	55	27	0	0
Glutathione	96	58	40	9	0	0

the wood-rotting fungus, *P. versicolor*. It occurs also in the mycelium of *Piricularia;* the *Polyporus* mycelium has not been investigated.

Relation of Thiooxidase to Polyphenoloxidases—Although the phenol-oxidases and thiooxidase usually occur together in the culture medium, the sulfhydryl-oxidizing and phenol-oxidizing activities appear to belong to distinct enzymes. The thiooxidase is demonstrable as early as 65 hours (Table II), whereas the laccase and metapolyphenoloxidase do not appear until considerably later. As the cultures age, the ratios thiooxidase-laccase and thiooxidase-metapolyphenoloxidase change markedly.

Furthermore, the several enzymes are not equally sensitive to inhibitors. Thus, 10^{-5} M azide inhibited both laccase and metapolyphenoloxidase 50 per cent but decreased the activity of thiooxidase by only 25 per cent.

Thiooxidase activity has been eliminated completely from some preparations by fractional precipitation of the phenoloxidases with acetone and ammonium sulfate.

Substrate Specificity—Besides the dialkyl dithiocarbamates, the following substances were oxidized by thiooxidase: alkylxanthates (R = methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, or *n*-amyl), thioacetate, dithioacetate,

TABLE II

Time-Course of Appearance of Extracellular Thiooxidase, Laccase, and Metapolyphenoloxidase in Culture of P. oryzae

Age of culture	Activity, μl. of O_2 per hr. per ml.			Ratio, $\frac{\text{laccase}}{\text{thiooxidase}}$
	Thiooxidase*	Laccase†	Metapoly-phenoloxidase‡	
hrs.				
65	12	0	0	0
96	16	0	0	0
139	320	198	67	0.62
168	470	390	149	0.83
240	603	605	289	1.01
288	690	680	320	0.99

* Substrate 0.0167 M sodium diethyldithiocarbamate, pH 6.0.
† Substrate 0.043 M hydroquinone, pH 4.0.
‡ Substrate 0.086 M resorcinol, pH 7.0.

dithiooxalate, thioglycolate, thiophenol, *p*-bromophenylthiopseudourea, 2-mercaptobenzothiazole, 5-amino-2-benzimidazolethiol, thiohistidine, and ergothioneine. The following were not oxidized: *n*-butyl mercaptan, mercaptoethylamine, thioglycerol, mercaptosuccinic acid, cysteine, and glutathione. Obviously the structural requisite for oxidizability is not merely the possession of a sulfhydryl group. With the exception of thioglycolic acid, which is oxidized rather slowly, all the compounds which are oxidized by the enzyme possess a sulfhydryl group attached to a carbon atom which is linked by a double bond to another carbon, nitrogen, oxygen, or sulfur atom:

DISCUSSION

Apart from the claim of Bertrand and Gavard (2, 3), which has been refuted by Tissières (4), that cysteine is oxidized to cystine by laccase, the only report of direct enzymatic oxidation of sulfhydryl compounds to disulfides is that of Mandels (5). He described the occurrence in spores of *Myrothecium verrucaria* of an enzyme capable of oxidizing cysteine, glutathione, homocysteine, and thiophenol to the corresponding disulfides. This enzyme, moreover, was unaffected by cyanide, azide, and DEDTC, was inhibited by thioglycolate, and did not catalyze the oxidation of thioacetic acid. Hence it appears entirely unrelated to thiooxidase.

The role of thiooxidase is at present entirely conjectural; it is not inconceivable that it may function as a terminal oxidase. According to Ward (6), the slime mold *Physarum polycephalum* contains an atypical ascorbic acid oxidase which is stimulated by DEDTC and TETDS but not by cysteine or glutathione. He suggested that these compounds may act as models of some unknown carrier which functions by shuttling between the —SH and S—S forms. Thiooxidase might be presumed to participate in the oxidation and reduction of such a carrier.

In this connection it is of considerable interest that TETDS has been shown recently to occur in the mushroom *Coprinus atramentarius* (7), suggesting the possibility that diethyldithiocarbamate itself may also be of natural occurrence. Conceivably DEDTC and TETDS might be not merely models in Ward's system but the actual carriers. Enzymatic reduction of thiuram disulfides both by fungi and by animal tissue has been reported (8–11).

The oxidizability of ergothioneine by thiooxidase suggests the possibility also that this compound, which is of widespread occurrence in plant and animal tissue but of presently unknown role, may function in such an oxidation system.

SUMMARY

Cell-free culture filtrates of *Piricularia oryzae* and *Polyporus versicolor* contain an enzyme which catalyzes the direct oxidation by atmospheric oxygen of compounds containing the structure $\geqslant$C—SH. Oxidizable substrates include dithiocarbamates, alkylxanthates, thioacetate, dithioacetate, dithiooxalate, thiophenol, thioglycolate, thiohistidine, and ergothioneine, among others. The dithiocarbamates are oxidized to the corresponding disulfides. Butyl mercaptan, thioglycerol, cysteine, glutathione, and mercaptosuccinic acid are not oxidized.

The new enzyme, which has been designated "thiooxidase," appears to be distinct from previously described enzymes that effect oxidation of sulfhydryl compounds.

We are indebted to Mr. George Svarnas and Mr. Freeman Young for synthesizing the sodium dimethyldithiocarbamate and potassium dithioacetate, to Mr. David Stefanye for determining the infrared spectra, and to Dr. Robert C. Baldridge for generously supplying the ergothioneine. We wish also to acknowledge the assistance of Mr. Jules J. Weisler, Mr. Bertram W. Fuhr, and Mr. Richard A. Pamplin with some of the experiments.

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PORPHYRIN BIOSYNTHESIS IN ERYTHROCYTES

I. FORMATION OF δ-AMINOLEVULINIC ACID IN ERYTHROCYTES*

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(Received for publication, August 21, 1957)

On the basis of studies with duck erythrocytes (1–3), chicken erythrocytes (4–10), *Chlorella* (11), and those to be reported here, the enzymes involved in porphyrin biosynthesis may be divided into three groups. The first group contains a number of enzymes which include those of the citric acid cycle. These enzymes convert glycine and succinate or derivatives of these compounds to δAL.[1] The second group of enzymes is soluble and converts δAL to COPRO'gen (10). The third group is bound to cell particulates and converts COPRO'gen to PROTO (12).

This paper presents studies on whole cells and homogenates of chicken erythrocytes to delineate the enzymes and coenzymes that are involved in the synthesis of δAL. A simple and sensitive method for the determination of protoporphyrin synthesis is described with which it was possible to examine a large number of substrates, inhibitors, and conditions in attempts to characterize the "active glycine" (13–15) and "active succinate" (3, 13) involved in δAL synthesis. The data obtained are also of interest for the information they provide on the general metabolism of chicken erythrocytes, *i.e.* the presence or absence of various enzymes and the coenzymes which under certain conditions become limiting. Previous scattered findings on materials from a number of species have been confirmed and extended with this readily available material.

The method of following porphyrin synthesis by determination of the PROTO synthesized is based on the finding (16) that when glycine is incubated with rabbit reticulocytes a marked increase in PROTO is observed. In chicken erythrocytes supplied with glycine the porphyrin synthesized was found to consist of about one-third newly formed heme and two-thirds PROTO (5). In order to investigate differential effects on porphyrin synthesis prior to δAL or steps beyond δAL, various compounds

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, R.G.-4922.

[1] Abbreviations used are as follows: δAL = δ-aminolevulinate; COPRO = coproporphyrin; COPRO'gen = coproporphyrinogen; PBG = porphobilinogen; PROTO = protoporphyrin; URO'gen = uroporphyrinogen; URO = uroporphyrin; ϵ = (1/cm. × mole per liter) × log I_0/I; mμmole = 10^{-9} mole.

have been examined in combination with glycine as substrate on the one hand, and with δAL as substrate on the other.

Methods

"Standard Conditions" for Preparation and Incubation of Whole Cells for Porphyrin Synthesis—The red cells from citrated chicken blood were washed twice with cold 0.25 M sucrose. The packed cells, approximately one-third the volume of the original blood, were suspended in an equal volume of a solution containing 0.07 M phosphate buffer, pH 7.5, 0.25 M sucrose, and 0.4 mg. of streptomycin per ml. For each experiment 5 ml. of this suspension were diluted with the designated reagents to a total volume of 5.6 ml., placed in a 30 ml. test tube stoppered with cotton, and incubated at 38° on a shaker for 15 hours. Because of the variable behavior of different batches of blood, it was found useful to incubate a large number of tubes from the same batch; the tubes were then preserved at −20° for 1 to several days prior to analysis.

Preparation of Red Cell Hemolysates by Freezing and Thawing—To 100 ml. of packed washed red cells were added 36 ml. of sucrose (0.5 gm. per ml.), 80 mg. of streptomycin, and 2 ml. of salt mixture (0.06 gm. of NaCl, 1.05 gm. of KCl, 0.016 gm. of $MgSO_4 \cdot 7H_2O$ per ml.). The suspension was rapidly frozen at −40° and then rapidly thawed. The mixture was diluted to 200 ml. with 0.05 M phosphate buffer, pH 7.4. The cells were completely hemolyzed.

Extraction of Porphyrins—A simplification of the method of Grinstein and Watson (17) was used. The incubated mixture was extracted three times, each time with 20 ml. of ethyl acetate-acetic acid (3:1 v/v) followed by centrifugation. The combined extract was neutralized with sodium acetate and washed with water, and the porphyrin was extracted from the ethyl acetate layer with a total of 5 ml. of 2.5 N HCl. The aqueous acid solution was washed with ether and diluted to 10 ml. with ethanol. Absorbancy was measured in the regions between 548 and 556 mμ or 400 and 409 mμ. Only porphyrins containing four or fewer carboxyl groups are extracted by this procedure. Positions of absorption maxima allow distinctions between COPRO (λ_{max} 401, 548 mμ) and PROTO (λ_{max} 409, 556 mμ). For further characterization the HCl numbers and paper chromatography with lutidine (18) were used. By this procedure recoveries from solutions containing 50 to 350 mμmoles of PROTO were as follows: from 5 ml. of aqueous buffer, 87 ± 4 per cent; from 5 ml. of a red cell suspension that had been preincubated at 38° for 15 hours, 72 ± 3 per cent; from 5 ml. of a red cell suspension kept at 38° for 15 hours, 56 ± 5 per cent. The PROTO used was purified by two crystallizations from formic acid-ethyl acetate and then from pyridine-chloroform.

"PROTO synthesized" designates the porphyrin mixture isolated by the above procedure from 2.5 ml. of packed red cells. As found by Dresel and Falk (5) the extracted porphyrin is PROTO with less than 10 per cent COPRO. The molar extinction coefficients, ϵ, used are those of PROTO, 1.4×10^4 at 556 mμ and 2.6×10^5 at 409 mμ.

Results with Whole Chicken Red Cells

Effect of Glycine on PROTO Synthesis—With increasing glycine concentration a typical saturation curve is obtained (Fig. 1) with an apparent Michaelis constant, K_m, of 0.02 M. From the curve of Dresel and Falk (4) with whole chicken blood the apparent K_m is estimated to be 0.005 M. The total porphyrin formed at maximal glycine concentrations is approximately the same as that found by these authors, based on an

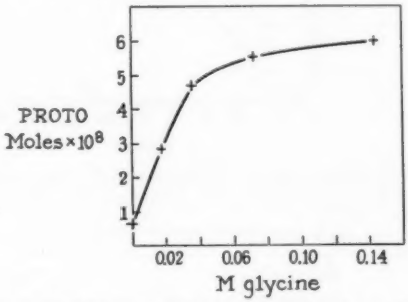

FIG. 1. Synthesis of PROTO *versus* glycine concentration. Incubation, 10 hours. Other conditions standard (see under "Methods").

equal volume of cells; the difference in the shape of the curve may be due to components of the serum which affect or supply substrates.

Rate of Porphyrin Synthesis—With glycine (0.07 M) and α-ketoglutarate (0.01 M) as substrates, the rate curve shows an induction period of about 2 hours and then is constant over a period of 18 hours in this case (Fig. 2). The absorption maxima of the porphyrin isolated were those of PROTO even after 2 hours incubation. The effect of increasing the temperature from 32–38° is very marked, corresponding to a $Q_{10} \cong 10$ in this range (Fig. 3). Whether this is due to a permeability effect, *e.g.* as a result of the melting of a lipide phase in the membrane, or to the activation of several rate-limiting processes will require further experiments to decide. At 45° the synthesis stopped after 8 hours although the red cells were not appreciably hemolyzed at this time.

Effect of Various Amino Derivatives Related to Glycine—For porphyrin synthesis only glycylglycine and ethyl glycinate could replace glycine

presumably by enzymic hydrolysis to glycine (Table I). Acetylglycine and synthetic β-alanine were slightly active. The following compounds

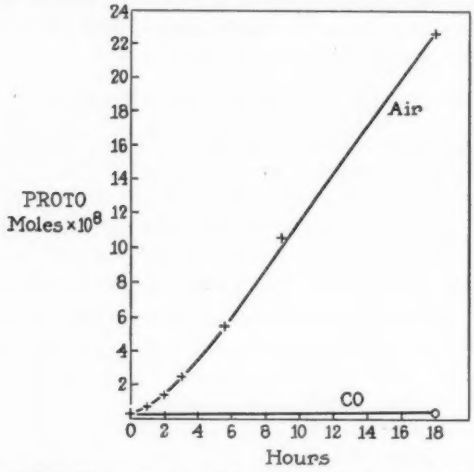

FIG. 2. Rate of PROTO synthesis. Substrates: 0.07 M glycine plus 0.01 M α-ketoglutarate. Incubation in air and in CO. Other conditions standard.

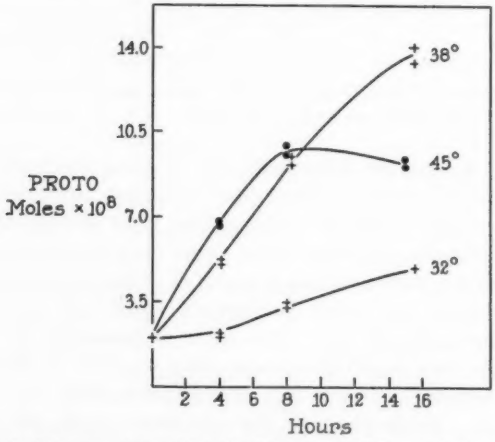

FIG. 3. Effect of temperature of incubation on PROTO synthesized. Substrates: 0.07 M glycine plus 0.01 M α-ketoglutarate. Other conditions standard.

at 0.02 to 0.075 M were inactive (6.3 to 7.9 mμmoles of porphyrin): diketopiperazine, hippuric acid, methylamine, *N*-methylglycine, betaine, creatine, creatinine, L-cysteine, L-serine, L-proline, L-glutamine, L-alanine. Casein

hydrolysate, although added in an amount sufficient to give a glycine concentration of 0.01 M, showed only slight activity which suggested that the presence of other amino acids might interfere with the synthesis of PROTO from glycine. The following amino acids were found to inhibit

TABLE I

Effect of Some Amino Derivatives Related to Glycine on Porphyrin Synthesis

Substrates were 0.01 M α-ketoglutarate plus the N compound. Other conditions were standard.

N compound	Concentration	Porphyrin
	M	*mμmoles*
β-Alanine	0.075	11.8
Casein hydrolysate	(0.13 gm.)	8.7
Glutathione	0.045	8.7
Acetylglycine	0.038	11.4
Glycine ethyl ester	0.057	49.3
Glycylglycine	0.037	66.5
Glycine	0.075	108

TABLE II

Inhibition of PROTO Synthesis by Certain Amino Acids

Substrates were 0.035 M glycine plus 0.01 M α-ketoglutarate plus the amino acid in the concentration shown. Other conditions were standard.

Amino acid	Concentration	Yield of PROTO
	M	*per cent of maximum*
L-Cysteine	0.035	5
	0.0087	43
	0.0043	75
L-Serine	0.035	52
	0.017	75
	0.0087	85
L-Arginine	0.035	56
L-Proline	0.035	64
	0.017	80
L-Alanine	0.035	70
	0.017	79

PROTO synthesis: L-cysteine > L-serine > L-arginine > L-proline > L-alanine (Table II). These amino acids did not inhibit PROTO synthesis when δAL was used as substrate. Therefore these amino acids inhibit some reaction prior to condensation of δAL to PBG. L-Aspartic acid, L-asparagine, L-glutamate, L-threonine, and DL-valine did not affect PROTO synthesis at concentrations of 0.035 M.

Effect of Citric Acid Cycle Substrates and Related Compounds—The exist-

ence of a complete citric acid cycle in duck erythrocytes was shown by the labeling experiments of Shemin and Kumin (3, 19) and of Wriston, Lack, and Shemin (20) and by metabolism experiments on chicken erythrocytes by Rubinstein and Denstedt (21).

To obtain further information on this cycle a number of compounds were tested for their effect on PROTO synthesis. In the absence of glycine none of the members of the citric acid cycle was found to enhance PROTO formation above that of the blank (7.9 mμmoles of PROTO). Glycine appears to be the limiting substrate. In the presence of glycine all members of the citric acid cycle at concentrations of 0.01 M with the

TABLE III

Effect of Some Carboxylic Acids on PROTO Synthesis in Presence of 0.07 M Glycine

Other conditions were standard.

Compound (0.01 M)	mμmoles PROTO formed	
	Cells of 1st batch	Cells of 2nd batch
None	58.0	34
Citrate	68.5	
α-Ketoglutarate	73.0	102
Succinate	71.0	96
Fumarate	86.8	100
L-Malate	71.8	103
Oxalacetate	39.4	
Pyruvate	34.6	
α-Ketobutyrate	33.0	
Maleate	7.1	
trans-Aconitate	7.5	

exception of oxalacetate were found to enhance PROTO formation. According to the batch of cells used the increase of porphyrin synthesis ranged from 30 to 300 per cent (Table III). The variability probably reflects the cell's own content of members of the citric acid cycle. The concentration of α-ketoglutarate or of succinate which resulted in a maximal yield of PROTO was about 5×10^{-3} M (Fig. 4). No effect was observed with acetate, L-glutamate, L-glutamine, L-aspartate, L-asparagine, or with L-tartrate at 0.01 M.

trans-Aconitate, an inhibitor of aconitase (22), and maleate were both found to be markedly inhibitory at 0.01 M (Table III). Both pyruvate and α-ketobutyrate at 0.01 M decreased PROTO formation by 40 per cent. Since added oxalacetate decomposes to pyruvate, this may account for its inhibition. Shemin *et al.* (23) have also observed inhibition with

uvate. Neither *trans*-aconitate, maleate, nor pyruvate inhibited the conversion of δAL to porphyrin; therefore, they act at a stage prior to δAL. The inhibition by 0.01 M pyruvate or α-ketobutyrate could be completely overcome by 0.005 M α-ketoglutarate.

Effect of Inhibitors Malonate, Arsenite, and Fluoroacetate—To localize the step in the formation of "active succinate" the action of some inhibitors was examined. Malonate inhibition of heme synthesis in duck erythrocytes was studied by Shemin and Kumin (3) with methylene- and carboxyl-labeled succinate; they found that in 0.02 M malonate, "active succinate" could be formed without going around the citric acid cycle to

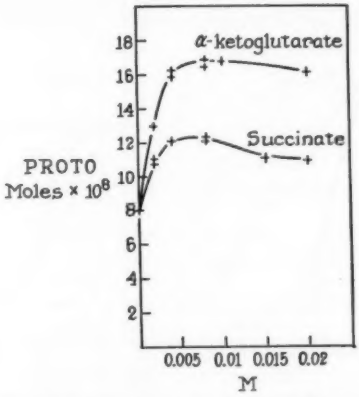

FIG. 4. Effect of increasing concentrations of α-ketoglutarate or of succinate on PROTO synthesis. Substrates: 0.07 M glycine plus indicated concentrations of α-ketoglutarate or succinate. Other conditions standard.

α-ketoglutarate, since the yield of labeled heme from methylene-labeled succinate in the presence of malonate was one-third that obtained in its absence. It was thus postulated that active succinate could be formed from either α-ketoglutarate or succinate.

In the present experiments with chicken erythrocytes incubated with glycine, 0.01 M malonate depressed PROTO formation to 10 per cent of the control. Addition of members of the citric acid cycle at 0.01 M partially overcame the malonate inhibition. α-Ketoglutarate increased the yield to 15 per cent of the control, succinate to 20 per cent, fumarate to 35 per cent, and malate to 50 per cent. Malonate did not inhibit the conversion of δAL to PROTO; therefore, malonate inhibits steps prior to δAL. The malonate inhibition curves are shown in Fig. 5, Curves D. The almost complete inhibition by malonate of PROTO synthesis in the

presence of α-ketoglutarate and glycine suggests that malonate may not only inhibit succinic dehydrogenase but may also combine with coenzyme A (24). Fluoroacetate inhibited PROTO formation from glycine even in the presence of α-ketoglutarate or succinate (Fig. 5, Curves C) or citrate, but did not do so with δAL as substrate. Fluoroacetate inhibition of the citric acid cycle has been explained on the basis that fluorocitrate is formed which poisons aconitase (25). The low slope of the curve may be due to an induction period required to form fluorocitrate. Arsenite inhibited PROTO formation from glycine and members of the citric acid cycle but not from δAL. The tailing of the curve for arsenite inhibition in the

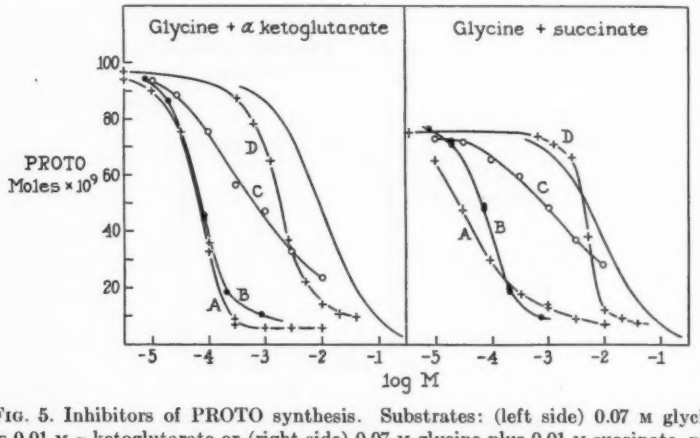

FIG. 5. Inhibitors of PROTO synthesis. Substrates: (left side) 0.07 M glycine plus 0.01 M α-ketoglutarate or (right side) 0.07 M glycine plus 0.01 M succinate, plus inhibitors at the indicated concentrations. Other conditions standard. The unmarked curves represent a simple reversible enzyme inhibition. Inhibitor curves: A, arsenite; B, dinitrophenol; C, fluoroacetate; D, malonate.

case of succinate (Fig. 5, Curves A) as compared to that of α-ketoglutarate may indicate a path for succinate activation independent of α-ketoglutarate. Arsenite is thought to inhibit α-keto acid oxidation by combining with lipoic acid.

Effect of O_2 and Dinitrophenol—Dresel and Falk (5) reported inhibition of porphyrin formation under N_2. In the present experiments porphyrin formation was inhibited completely by CO when glycine plus α-ketoglutarate, succinate, or fumarate was the added substrate. Since the conversion of δAL to COPRO'gen proceeds in the absence of O_2 (26), it is the formation of δAL which requires aerobic conditions. Dinitrophenol was found to inhibit when either succinate or α-ketoglutarate was the substrate (Fig. 5, Curves B). The inhibition curves follow that of a

reversible enzyme-inhibition curve. Dinitrophenol did not inhibit the conversion of δAL to PROTO. Dresel and Falk (5) have also observed the inhibition of porphyrin synthesis by dinitrophenol. These results suggest that ATP may be necessary for δAL formation but not for steps beyond δAL.

Other Inhibitors—The inhibition of porphyrin synthesis by a number of other compounds is given in Tables IV and V. The inhibition by 10^{-3} M azaserine was unaffected by 10^{-3} M pyridoxal phosphate, 0.02 M trypto-

TABLE IV

Other Inhibitors of PROTO Synthesis

Substrates were 0.07 M glycine plus 0.01 M α-ketoglutarate. Other conditions were standard.

Compound	Concentration	PROTO	Compound	Concentration	PROTO
	M	*per cent of maximum*		M	*per cent of maximum*
Na_2SO_3	0.05	8	Tryptamine	0.005	70
	0.01	14	Indoleacetate	0.01	18
	0.005	48		0.005	56
$CuSO_4$	0.001	40	Dimedon	0.001	87
$CoCl_2$	0.001	50	Hydroxylamine	0.01	11
$HgCl_2$	0.001	73		0.001	77
α,α′-Dipyridyl	0.01	90	Formaldehyde	0.005	85
o-Phenanthroline	0.001	44	Azaserine	0.001	40
NaF	0.01	61	Isonicotinic hydrazide	0.001	50
	0.001	100	Aminopterin	0.0005	80
Iodoacetamide	0.01	37	A-Methopterin	0.0005	100
	0.001	85	Benzimidazole	0.002	63
p-Chloromercuribenzoate	0.001	100	2-Ethyl-5-methylbenzimidazole	0.002	100
Levulinate	0.005	58	5,6-Dichloro-1-d-ribofuranosylbenzimidazole	0.006	100
Benzedrine sulfate	0.002	65			

phan, or 0.02 M phenylalanine. However, glutamine partially overcame the inhibition (Fig. 6). Azaserine has been found to act as a glutamine antagonist in the formation of formylglycine amidine ribotide (27, 28). Azaserine did not inhibit δAL conversion to porphyrin. Isonicotinic hydrazide is inhibitory as found by Larsen and Orten (29). Incubation with isonicotinic hydrazide led to the formation of a pale green pigment with a diffuse absorption spectrum which could be extracted from ethyl acetate with 2 N HCl. The inhibition by 10^{-3} M isonicotinic hydrazide could be overcome by 10^{-4} M pyridoxal phosphate. Isonicotinic hydrazide inactivates pyridoxal phosphate enzymes (30).

The requirement of pyridoxal phosphate for δAL synthesis by duck red cells was established by Schulman and Richert (31). Inhibitor ex-

TABLE V

Inhibition of PROTO Synthesis by Deozypyridozine and Reversal by Pyridozal Phosphate

Substrates were 0.07 M glycine plus 0.01 M α-ketoglutarate. Other conditions were standard.

Deoxypyridoxine	Pyridoxal-5-PO_4	PROTO
$M \times 10^5$	$M \times 10^5$	*mμmoles*
0		170
5		130
25		120
50		97
100		83
200		25
100	50	170
100	25	160
100	5	130

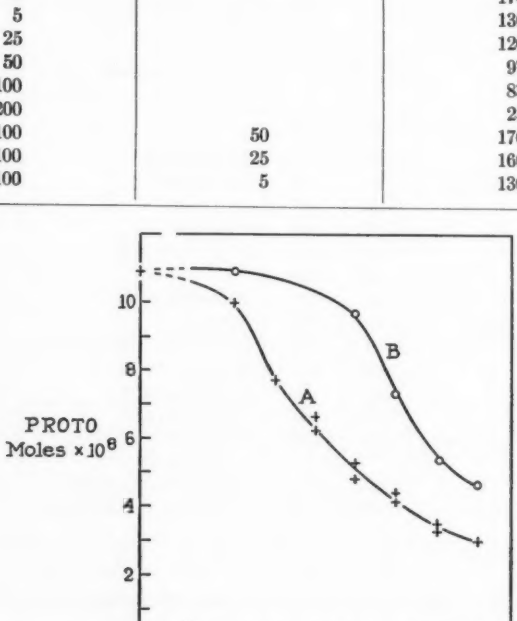

FIG. 6. Inhibition of PROTO synthesis by azaserine and reversal by glutamine. Curve A, substrates: 0.07 M glycine plus 0.01 M α-ketoglutarate plus azaserine at the indicated concentrations. Curve B, substrates: the same as Curve A plus 0.01 M glutamine. Other conditions standard.

periments with normal and hemolyzed chicken red cells confirm this result. Deoxypyridoxine inhibited porphyrin synthesis by 50 per cent at 10^{-3} M;

approximately 5×10^{-5} M pyridoxal 5-phosphate could reverse this inhibition (Table V). With δAL or PBG as substrate, porphyrin synthesis was not inhibited even by 5×10^{-3} M deoxypyridoxine. Compounds related to pyridoxal phosphate were also tested with glycine and α-ketoglutarate as substrates. At 5×10^{-3} M pyridoxamine was not inhibitory but pyridoxal decreased the PROTO yield by 34 per cent and pyridoxine by 17 per cent. Pyridoxal (5×10^{-4} M) or 10^{-4} M pyridoxine could overcome the inhibition of 10^{-3} M deoxypyridoxine; higher concentrations of pyridoxal or pyridoxine were less effective because they themselves became inhibitory.

Benzimidazole derivatives such as 2-ethyl-5-methylbenzimidazole have been reported (32) to inhibit incorporation of N^{15}-glycine into the heme of chicken red cells. Related compounds have been found to inhibit virus multiplication. Of the three benzimidazole compounds tested (Table IV) only benzimidazole itself was found to inhibit PROTO synthesis. The following compounds had no observable effect: 3×10^{-4} M thyroxine, 10^{-2} M pantothenate, 10^{-2} M inositol, 5×10^{-3} M Chloromycetin, 5×10^{-3} M histamine.

Results with Hemolyzed Chicken Red Cells

This section includes studies on methods of hemolysis designed to retain some porphyrin-synthesizing ability. The method of choice proved to be freezing and thawing the cells. A standard preparation of frozen and thawed cells as described under "Methods" was used to examine conditions for reactivating the system which carries out PROTO synthesis.

Decrease in Porphyrin Synthesis on Hemolysis—When packed cells, previously washed with isotonic sucrose, were diluted with varying volumes of water, the results shown in Table VI were obtained. 3 volumes of water were required to hemolyze all the cells in 10 minutes. When packed cells were frozen and thawed once, all the cells were hemolyzed. The addition of the salt mixture prevents the nuclei from breaking down and forming a viscous gel in the hemolysate. The activity of this hemolysate for PROTO synthesis in the presence of 0.07 M glycine as added substrate was about 20 per cent of that of an unhemolyzed control. This hemolysis method gave more reproducible results than hemolysis with water. Cells hemolyzed with ether or by ultrasonic vibrations had negligible activity.

Partial damage to red cell permeability may be brought about with digitonin. 1 mg. of digitonin per 5.6 ml. of cell suspension gave about 50 per cent hemolysis by the end of the incubation period and decreased the PROTO yield to 72 per cent when glycine (0.07 M) plus α-ketoglutarate (0.01 M) were the substrates; with 2 mg. of digitonin (75 per cent hemolysis) the yield was 58 per cent, and with 4 mg. (100 per cent hemolysis)

33 per cent. Incubation in the presence of 1 mg. of digitonin resulted in a 3-fold increase of porphyrin from δAL or PBG compared to controls without digitonin.

The above results indicate that steps in the synthesis from glycine to δAL may require coenzymes which become diluted out on damage to the cell membrane, or may require enzymes which are readily damaged on cell hemolysis; whereas steps of porphyrin synthesis from δAL to PROTO are not critically damaged by hemolysis. A permeability barrier to δAL and PBG is also evident.

TABLE VI

Effect of Hemolysis with Water on PROTO Synthesis

Washed packed cells were diluted with different volumes of water. After 10 minutes at 25°, 2.5 ml. of sucrose containing M/15 phosphate buffer, pH 7.4, were added to 2.5 ml. of the mixture to make the preparation isotonic with respect to sucrose. Substrates were 0.07 M glycine plus 0.01 M α-ketoglutarate. Incubation and other conditions were standard. The yield of protoporphyrin was calculated on the basis of 2.5 ml. of packed cells.

Ratio, $\frac{\text{volume packed cells}}{\text{volume } H_2O}$	Protoporphyrin
	moles × 10^9
Unhemolyzed control minus substrates	0.20
" " plus "	12.5
1:1	8.88
1:2	1.07
1:3	0.87
1:4	0.59

Enhancement of PROTO Synthesis by Substances Incubated with Hemolysates of Frozen and Thawed Cells—Dresel and Falk (4) reported that an extract of boiled bakers' yeast increased porphyrin synthesis of hemolyzed chicken red cells up to 6-fold. Shemin and Kumin (3) found that a boiled liver extract, or Armour's coenzyme A concentrate, enhanced heme synthesis by hemolyzed duck red cells; they considered that this effect was possible evidence for the participation of succinyl coenzyme A in heme synthesis.

Many substrates and coenzymes were tested on frozen and thawed hemolysates of chicken red cells for their effect on porphyrin synthesis. The cell preparations varied in activity over a 2-fold range, and only results from the same batch are comparable. In general, glycine was required for any observable increase in PROTO synthesis. Members of the citric acid cycle did not affect PROTO formation. An increase in PROTO synthesis depended on the simultaneous presence of certain compounds.

For example, inosine and glutamine enhanced the yield only when added together and then only if glycine and pyridoxal phosphate had also been added. The order of additions which gave progressive increases in porphyrin is as follows: 0.07 M glycine, 5×10^{-4} M pyridoxal phosphate, 1×10^{-4} M coenzyme A, 5×10^{-4} M diphosphopyridine nucleotide, 5×10^{-3} M glutamine, and 5×10^{-3} M inosine. The PROTO formed on aerobic incubation of 5 ml. of the hemolysate for 15 hours at 38° was 4 mμmoles without added substrates and 23 mμmoles with all the above substrates. The yield with added substrates was 35 per cent of the PROTO yield obtained with non-hemolyzed cells incubated with glycine.

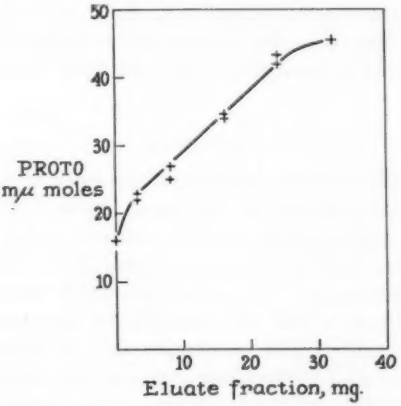

FIG. 7. PROTO synthesis by 5 ml. of frozen and thawed chicken red cell hemolysate. Substrates: 0.07 M glycine, 5×10^{-4} M pyridoxal phosphate, and fraction from pig liver. Aerobic incubation for 15 hours at 38°.

An increase of PROTO to 63 per cent of that obtained with intact cells incubated with glycine was obtained with frozen and thawed cells incubated with the following mixture: 26 mg. of glycine, 0.5 mg. of pyridoxal phosphate, 0.3 mg. of coenzyme A, 5.0 mg. of glutamine, 2 mg. of ribose 5-phosphate, 2 mg. of diphosphopyridine nucleotide, 1 mg. of adenosine triphosphate, 0.5 mg. each of uridine and guanosine triphosphates, and uridine, adenosine, guanosine, and cytidine diphosphates, 0.6 mg. of cytochrome *c*, 1.0 mg. of diphosphopyridine nucleotide cytochrome *c* reductase, and a phosphate buffer (pH 7) extract from 1 mg. of acetone powder of pigeon liver.

From pig liver a fraction has been isolated which, when incubated with a frozen and thawed red cell hemolysate together with glycine and pyridoxal phosphate, gives a maximal yield of PROTO equivalent to 80 per cent of

that of non-hemolyzed red cells incubated with glycine. Ground pig liver was deproteinized with 0.6 N perchloric acid; the extract was adjusted to pH 3, adsorbed on acid-washed Nuchar, and eluted with acetone-ammonia (33). The eluate fraction, pH 4 to 5, had most of the activity. Incubation of the hemolysate with this fraction gave a progressive increase in PROTO over a period of 15 hours. Dinitrophenol or arsenite at 10^{-3} M completely inhibited the synthesis. The hemolysate alone formed 8 mμmoles of PROTO, with added glycine it formed 11.5 mμmoles, and with further addition of pyridoxal phosphate and the liver fraction it formed a maximum of 46 mμmoles of PROTO (Fig. 7).

Particulate cell components are required for porphyrin synthesis from glycine. When frozen and thawed solutions of red cells were centrifuged for 1 hour at 20,000 × *g*, the supernatant solution, incubated together with glycine, pyridoxal phosphate, and the liver fraction, did not form porphyrin.

DISCUSSION

The present studies confirm and extend the work of other investigators, suggesting that the synthesis of δAL from glycine (Fig. 8) requires an electron transfer system to O_2, coupled with oxidative phosphorylation to form ATP, a citric acid cycle system to form active succinate, pyridoxal phosphate, and glutamine. The cofactors which have been found to enhance δAL synthesis in red cell homogenates are pyridoxal phosphate, coenzyme A, diphosphopyridine nucleotide, and, in addition, some factors that are present in protein-free liver extracts and which are in part replaceable by a mixture of known compounds. The electron transport system, oxidative phosphorylation, and the citric acid enzymes are known to be constituents of mitochondria. Centrifugation studies indicate that insoluble cell components are involved in δAL synthesis. One may therefore consider that mitochondria are active in δAL synthesis.

The requirement of vitamin B_6 in the production of normal red cells was clearly demonstrated by the work of Wintrobe (34) who found that pigs deficient in vitamin B_6 produced small pale red cells deficient in heme and with a low content of free protoporphyrin. The low heme and porphyrin are due to a pyridoxal phosphate requirement for δAL synthesis. A reasonable mechanism is the formation of a Schiff base between glycine or a derivative and pyridoxal phosphate, thus activating the methylene group to condense with "active succinate." The smallness of the cells indicates that their protein content is low possibly because pyridoxal phosphate was lacking to bring about transaminations which are required for globin synthesis.

Synthesis of δAL was found to be inhibited by azaserine and overcome

by glutamine. This fact suggests that glutamine is involved in δAL synthesis. Whether "active glycine" may be represented by a glycine intermediate of purine biosynthesis remains for study.

Control mechanisms which may be involved in δAL synthesis are suggested by the inhibitory effects shown by certain amino and keto acids. It is probable that the inhibition by cysteine, serine, etc., is not due to a competition for pyridoxal phosphate since aspartate and glutamate do not inhibit. Conceivably a partial block to δAL is removed when these amino acids reach a low concentration in the cell relative to glycine. Thus in

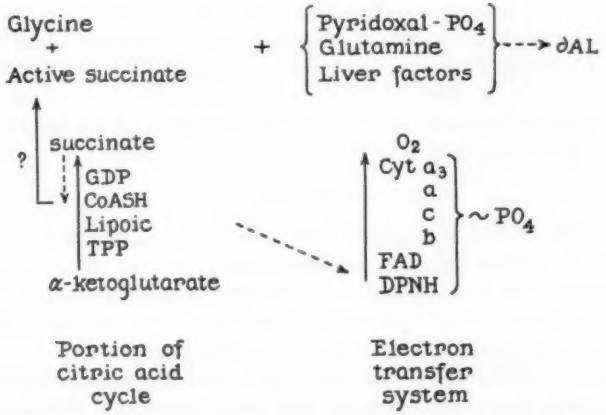

FIG. 8. Summary of the enzyme systems and factors that may be required for δAL synthesis from glycine and succinate, where GDP = guanosine diphosphate; CoASH = coenzyme A; TPP = thiamine pyrophosphate; FAD = flavin adenine dinucleotide; DPNH = reduced diphosphopyridine nucleotide; CYT = cytochrome; $\sim PO_4$ = high energy phosphate.

erythroblasts during rapid globin synthesis such amino acids might diminish to a level which permits rapid δAL synthesis. The result would be a coupling of globin with heme synthesis. Likewise, in certain *Chlorella* mutants (35) and in a strain of *Tetrahymena* (36) which synthesize PROTO, this pigment is found to accumulate most rapidly toward the end of the phase of rapid cell multiplication when protein synthesis has depleted the nitrogen supply of the medium. Pyruvate is an inhibitor of δAL synthesis. Shemin *et al.* (23) have suggested that pyruvate might compete with active succinate for glycine to form amino acetone. Since α-ketobutyrate was found to be as effective an inhibitor of δAL synthesis as was pyruvate, the suggested reaction is perhaps more general and includes the condensation of active glycine with a number of α-keto acids. However, competition

between these α-keto acids and α-ketoglutarate for coenzymes is also possible.

The conversion of δAL to PROTO is inhibited neither by cyanide nor by dinitrophenol. These results suggest that, although O_2 is required for COPRO'gen conversion to PROTO, oxidation through cytochrome oxidase and oxidative phosphorylation is not necessary for these latter steps.

The addition of a number of compounds to frozen and thawed red cell hemolysates permits continued synthesis of PROTO for 15 hours or longer. This result suggests that such compounds may be useful for the preservation of red cell metabolism during storage.

We wish to acknowledge our gratitude to Mrs. Annabelle Long and Mr. William Cumming for skilful technical assistance, to Dr. D. Mauzerall for advice, to Dr. K. Folkers of Merck and Company, Inc., for samples of benzimidazole compounds, and to Dr. Martin Black of Parke, Davis and Company for a sample of azaserine.

SUMMARY

1. A rapid method is described for the determination of protoporphyrin synthesis by chicken red cells and hemolysates.

2. Synthesis of δ-aminolevulinic acid was found to require at least a portion of the citric acid cycle with its coenzymes, an oxidative phosphorylating system, pyridoxal phosphate, and glutamine.

3. Various inhibitors of δ-aminolevulinic acid including certain amino and keto acids have been examined and explanations are offered for their action.

4. The simultaneous addition of a relatively large number of substances increased protoporphyrin synthesis in frozen and thawed chicken red cells up to 80 per cent of that of unhemolyzed cells.

Addendum—Recently both Gibson, Laver, and Neuberger (37) and Shemin, Kikuchi, and Bachmann (38) have reported the synthesis of δ-aminolevulinic acid from glycine, succinyl coenzyme A, and pyridoxal phosphate with preparations of anemic chicken erythrocytes and *Rhodopseudomonas spheroides* respectively. We have found that 6-diazo-5-oxo-nor-L-leucine does not inhibit δAL synthesis under conditions where azaserine did.

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PORPHYRIN BIOSYNTHESIS IN ERYTHROCYTES

II. ENZYMES CONVERTING δ-AMINOLEVULINIC ACID TO COPROPORPHYRINOGEN*

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(Received for publication, August 21, 1957)

Soluble enzyme preparations which convert δAL[1] or PBG to porphyrins have been obtained from a number of different animal and plant cells such as duck erythrocytes (1–3), chicken erythrocytes (4–6), *Chlorella* (7), and spinach (8). The enzymic conversion of δAL to PBG has been studied by Gibson, Neuberger, and Scott (9–11), by Granick (12), and by Schmid and Shemin (3). The enzymic conversion of PBG to URO'gen has been studied by Bogorad (8).

In this paper a method is described for the separation of three soluble enzyme fractions from red cells by zone electrophoresis. One enzyme fraction converts δAL to PBG (δAL-ase), another converts PBG to URO'gen III (PBG-ase), and a third fraction converts URO'gen to COPRO'gen (UD-ase). Some properties of the enzymes contained in these fractions will be discussed. A bulk method for the isolation of δAL-ase from chicken erythrocytes is also described. In the following paper additional properties of the enzyme fraction containing UD-ase will be considered.

EXPERIMENTAL

Compounds and Reagents Used—δAL was prepared by the pthalimide synthesis according to the procedure of Dr. J. Dice (personal communication). A generous sample was also obtained from Parke, Davis and Company. PBG was obtained from the urine of patients with acute porphyria and was purified by the method of Cookson and Rimington (13). Samples

* This is Paper II of a series on enzymes in porphyrin biosynthesis. This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, No. R.G.-4922.

[1] Abbreviations used are as follows: δAL = δ-aminolevulinic acid; PBG = porphobilinogen; URO = uroporphyrin; URO'gen III = uroporphyrinogen isomer III; COPRO = coproporphyrin; COPRO'gen = coproporphyrinogen; δAL-ase = enzyme that converts δAL to PBG; PBG-ase = enzyme that converts PBG to URO'gen; UD-ase = enzyme that converts URO'gen to COPRO'gen; supernatant fluid = supernatant solution obtained from red cells by centrifugation of the hemolyzed cells; Tris = tris(hydroxymethyl)aminomethane; GSH = reduced glutathione; TCA = trichloroacetic acid; EDTA = ethylenediaminetetraacetic acid; Γ/2 = ionic strength.

of natural URO and COPRO isomers were obtained from Professor C. Rimington. Synthetic samples of URO isomers were obtained from Dr. S. F. MacDonald. Bovine serum albumin was Armour's fraction V. Evans blue albumin marker was prepared by dissolving crystalline Armour's bovine albumin and 3 mg. of Evans blue dye in 5 ml. of Tris buffer, $\Gamma/2 = 0.05$, pH 7.65.

Electrophoretic Separation of Enzymes

The general method of separation is described for rabbit reticulocytes. These were obtained by injecting each of four rabbits intraperitoneally with 60 mg. of acetylphenylhydrazine dissolved in 10 ml. of isotonic saline. After 6 days, the two rabbits with the highest reticulocyte count (30 and 35 per cent) were bled from the heart. 5 ml. of 0.5 per cent heparin were used to prevent the clotting of 75 ml. of blood. The cells were washed by centrifugation in the cold room first with saline-citrate (100 ml. of 0.9 per cent NaCl plus 2 ml. of 10 per cent sodium citrate) and then twice with 0.25 M sucrose. A total of 50 ml. of packed cells was obtained. The packed cells were hemolyzed by shaking with 8 ml. of ether. After 5 minutes most of the ether was removed *in vacuo*. The hemolyzed material was centrifuged for 1 hour at 14,000 $\times$ *g*, yielding 40 ml. of supernatant solution.

The method of Kunkel (14) of zone electrophoresis on starch was used for the separation of enzymes contained in the supernatant fluid. The following modifications were found effective for large scale separation of material and the partial protection of enzyme activities.

Two starch plates were run simultaneously in parallel by means of a power unit containing a 1000 volt, 250 ma. transformer. Potato starch placed on a Büchner funnel was first washed with Tris buffer, pH 7.65, $\Gamma/2 = 0.05$. When semi-dry, the starch was mixed with 1 liter of 0.25 per cent bovine albumin dissolved in the same buffer and aspirated until no foaming was observed. The starch was mixed with the buffer and formed into two starch plates, each 2 cm. thick, 20 cm. wide, and 45 cm. long.

Two streaks of supernatant fluid were now applied to each starch plate at 7 cm. and 26 cm. distance from the cathode end (Fig. 1, origin). Each streak contained 7.5 ml. of supernatant fluid; thus a total of 30 ml. could be fractionated simultaneously. The electrophoresis was run at 90 ma., 270 volts, for 27 hours at 5°. The potential drop across the starch plate was 3.1 volts per cm.

To observe the rate and uniformity of migration on the two starch plates, hemoglobin and Evans blue albumin were used as markers. At the end of the run the two hemoglobin streaks were 2 to 6 cm. and 20 to 25 cm.

distant from the cathode end. The Evans blue albumin spots were 19 ± 1 cm. and 38 ± 1 cm. distant from the cathode side, *i.e.* the mobility was about 0.15 $cm.^2$ $hour^{-1}$ $volt^{-1}$.

After electrophoresis the two starch plates were cut into 2 cm. wide segments, and identical segments of the two plates were combined and eluted with 40 ml. or more of 0.05 M Tris buffer. The eluates may be concen-

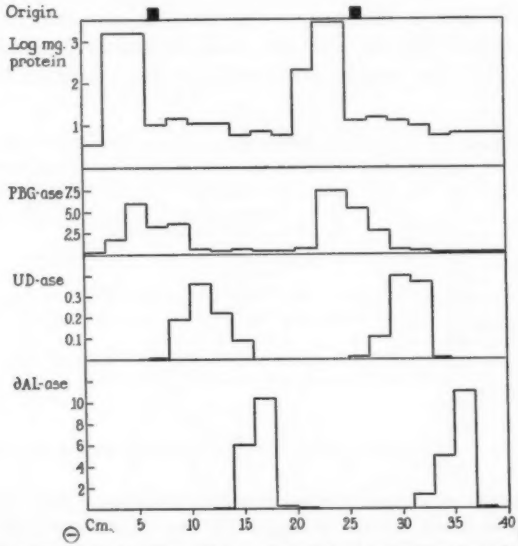


FIG. 1. Electrophoretic separation on starch of supernatant fluid from rabbit reticulocytes (see the procedure described in the text). The figures represent the total protein and total activity of eluates from 2 cm. wide segments of two identical starch plates. δAL-ase activity is expressed as micromoles of PBG formed, PBG-ase activity as micromoles of PBG converted, and UD-ase activity as micromoles of COPRO'gen formed, all per hour per fraction.

trated 10- to 20-fold by filtration at 5° through Schleicher and Schüll collodion bag ultrafilters. An eluate containing the rabbit δAL-ase was found to retain over 85 per cent of its activity when concentrated over 20-fold in this manner. Enzyme activities and protein concentrations were determined on aliquots of the eluates. Protein concentration was determined by the colorimetric procedure of Lowry *et al.* (15) and referred to a standard of crystalline bovine albumin. Methods for the determination of enzyme activities are considered in the experimental sections devoted to the particular enzymes.

The recovery of δAL-ase activity from the starch after electrophoresis

was increased from 48 up to 69 per cent when the starch was prewashed with bovine albumin, and from 8 up to 36 per cent in large scale (*i.e.* bulk) preparations.

Separation of Three Enzyme Fractions by Zone Electrophoresis—When the supernatant solutions of red cell hemolysates were subjected to zone electrophoresis, the main component, hemoglobin, migrated towards the cathode, whereas three colorless enzyme fractions concerned with porphyrin synthesis migrated towards the anode. One fraction converted δAL to PBG, another converted PBG to URO'gen, and the third converted URO'gen to COPRO'gen. The enzyme fractions have been isolated from super-

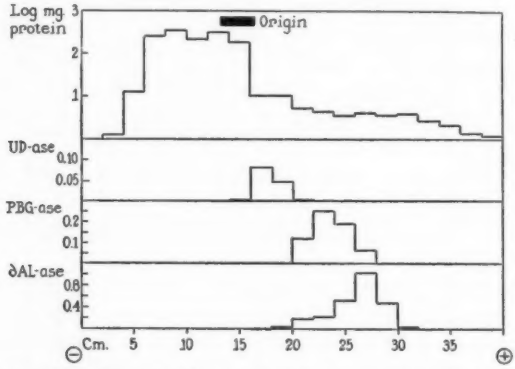


FIG. 2. Electrophoretic separation on starch of supernatant fluid from chicken erythrocytes. One streak of 5 ml. was applied to one plate at the origin. The run was made in phosphate buffer, pH 6.9, $\Gamma/2 = 0.13$, at 80 ma., 300 volts, for 19 hours at 5°. The figures represent the total protein and total activity of δAL-ase, PBG-ase, and UD-ase (see the legend to Fig. 1) per 2 cm. wide segment.

natant solutions of rabbit reticulocytes, of erythrocytes of the chicken (variety Plymouth Rock), and of immature red cells of a case of erythroblastosis fetalis of the human. The relative rates of migration are as follows: for the rabbit enzymes, δAL-ase > UD-ase > PBG-ase (Fig. 1); for the chicken, δAL-ase > PBG-ase > UD-ase (Fig. 2); and for the human, UD-ase > PBG-ase > δAL-ase (Fig. 3). The rabbit preparation yielded eluates from the starch which possessed the highest enzyme activities expressed in micromoles per hour per mg. of protein. The maximal activities were as follows: for δAL-ase, 0.8 μmole of PBG; PBG-ase, 0.44 μmole of PBG; and UD-ase, 0.009 μmole of COPRO'gen. The maximal activities for the chicken preparation were as follows: for δAL-ase, 0.3 μmole of PBG; PBG-ase, 0.05 μmole of PBG; and UD-ase, 0.007 μmole of COPRO'gen. The maximal activity of δAL-ase isolated by the bulk method was

1.6 μmoles of PBG. Gibson *et al.* (11) have reported a maximal activity for δAL-ase of about 3 μmoles for their beef liver preparation.

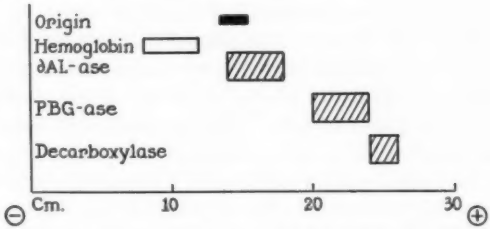


FIG. 3. Electrophoretic separation on starch of the supernatant fluid from 7 ml. of red cells from a case of erythroblastosis fetalis. The run was made in phosphate buffer, pH 6.9, $\Gamma/2 = 0.13$, at 5° for 20.5 hours at 85 ma., 350 volts. The qualitative distribution of the enzymes is presented.

TABLE I

Bulk Isolation Method of Chicken δAL-ase

Treatment	Fraction	Volume	Mg. N per ml. Kjeldahl	Activity in moles of PBG per hr. per mg. protein N $\times 10^7$	Per cent of starting activity
		ml.			
Hemolysate from 215 ml. packed cells	A	710	20.4	0.37	100
1st alcohol-$CHCl_3$ denaturation. Combined filtrate	B + C	860	2.13	2.74	94
2nd alcohol-$CHCl_3$ denaturation; filtrate	H	940	0.42	7.8	57
Acetone pptn.	J	61	1.56	22.5	41
Electrophoresis on starch	K	336	0.073	101	15

Bulk Method for Isolation of δAL-ase

Procedure for Bulk Isolation of δAL-ase from Chicken Erythrocytes—The hemoglobin is removed in two steps by denaturation with an alcohol-chloroform mixture. The filtrate containing the enzyme is concentrated by precipitation with acetone and the δAL-ase is separated by zone electrophoresis on starch. Chicken erythrocytes were washed free from citrated serum with saline and the buffy top layer was removed. The washed packed cells (215 ml.) were hemolyzed by adding 430 ml. of H_2O. After 5 minutes at 25°, 65 ml. of 1 M NaCl were added. The remainder of the operations were carried out at 5°. The hemolysate, Fraction A (Table I), was mixed in a blendor for 1 minute with alcohol-chloroform in the pro-

portion of 350 ml. of hemolysate to 40 ml. of alcohol-chloroform (3 volumes of ethanol, 95 per cent, to 1 volume of $CHCl_3$). The mixture was filtered by suction yielding 430 ml. of a reddish filtrate, Fraction B. The somewhat moist residue was resuspended in 0.05 M Tris buffer, pH 7.6, and refiltered yielding 430 ml. of filtrate, Fraction C. The reddish filtrates, Fractions B plus C, were combined, mixed in a blendor with alcohol-chloroform in the proportion of 400 ml. of filtrate to 40 ml. of the alcohol-chloroform solution for 1 minute, and filtered. Excess alcohol-chloroform should be avoided. The moist residue was washed with 40 ml. of Tris buffer and sucked dry. The total volume of filtrate, Fraction H, was 940 ml., pale yellow in color. To concentrate the protein, 300 ml. of acetone at $-20°$ were added to 200 ml. of filtrate, Fraction H; the flocculent precipitate was centrifuged immediately at $2000 \times g$ for 10 minutes and rapidly resuspended in 10 ml. of GSH-phosphate buffer (M/15 phosphate buffer, pH 6.8, containing 0.01 M GSH). 61 ml. of a brown solution, Fraction J, were obtained.

For electrophoresis, 10 ml. of Fraction J were placed as a streak on a starch plate that had been prewashed with bovine albumin and prepared with a phosphate buffer, pH 6.8, $\Gamma/2 = 0.13$. After electrophoresis for 18 hours, the starch segments were eluted with 0.025 M phosphate buffer, pH 6.8. Two segments contained 99 per cent of the recovered δAL-ase activity in a total volume (Fraction K) of 55 ml. This δAL-ase was further concentrated by filtration through a collodion bag ultrafilter.

Fractions B plus C may be used in the preparation of PBG from δAL. Not only is the δAL-ase activity relatively high but much of the PBG-ase has been destroyed at this step. In addition, it has been found that the presence of some hemoglobin prevents PBG from undergoing spontaneous decomposition. The δAL-ase enzyme at this stage requires no activation with GSH. When δAL at a concentration of 5×10^{-3} M was incubated anaerobically in this enzyme solution in 0.02 M phosphate buffer, pH 6.8, for 16 hours at 38°, colorimetric determination of PBG indicated a yield of 35 per cent of theory.

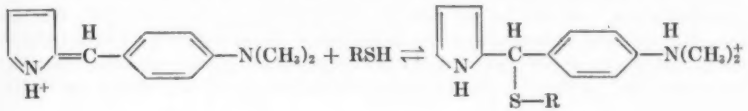
Properties of δAL-ase

Measurement of δAL-ase Activity—δAL-ase activity is defined as the micromoles of PBG formed in 1 hour per ml. of enzyme solution. Standard conditions for the measurement of the activity are the following. Enzyme solutions are activated by preincubation for 30 minutes in 0.01 M GSH at pH 6.8, 38°. To 0.2 ml. of activated enzyme solution are added 0.1 ml. M/15 PO_4 buffer, pH 6.8, and 0.1 ml. of neutral 0.01 M δAL. The solution is incubated for 1 hour at 38°. The activity is stopped by the addition of 1 ml. of a TCA-Hg mixture (80 ml. of 5 per cent TCA plus 20

ml. of 0.1 M $HgCl_2$). After centrifugation, 1 ml. of supernatant solution is removed and mixed with 1 ml. of 4 N perchloric Ehrlich reagent (16). The resulting color density is measured against a reagent blank at 553 mμ in a cell of 1 cm. optical length at 3 minutes after mixing. The activity = density × 0.226 μmole of PBG formed per hour per ml. of enzyme solution.

Preincubation with GSH for Activation—GSH was found to be required for the activation of beef liver δAL-ase (9) and for the δAL-ase of duck erythrocytes (3). Chicken and rabbit δAL-ase isolated by zone electrophoresis also require reactivation with GSH. The reactivation of rabbit δAL-ase is complete within 15 minutes at 38°. A 10-fold increase in activity of chicken δAL-ase has been observed on reactivation.

Interference of GSH in Determination of PBG with Ehrlich's Reagent—On allowing a pyrrole with a free α position to react with *p*-dimethylaminobenzaldehyde in acid solution, a pink Ehrlich color salt I arises (17). GSH and other sulfhydryl compounds decolorize this compound. Nucleophilic reagents such as R—SH would be expected to add at the methine carbon to produce the colorless compound II, which is protonated in acid solution. The reaction is analogous to the equilibrium between sulfite ions and dipyrrylmethenes given in the following paper (Paper III). Evidence for such a reaction was obtained by measuring its equilibrium constant, using



(I) (II)

compound I derived from the reaction of 2-methyl-3-acetyl-4-(3-propionic acid)-pyrrole and the 4 N perchloric-Ehrlich reagent. The dissociation constant, K, for compound II to form compound I plus GSH is about 3×10^{-5} M at room temperature. The exact value of K cannot be established due to the transient nature of compound I (readily apparent in the case of PBG as the pyrrole) and our ignorance of its absolute concentration (16). Further evidence for this equilibrium comes from the fact that removal of R—SH by oxidation or by forming tight complexes with heavy metals, either before or after addition of the Ehrlich reagent, produces the color salt I. Since Compound I is also sensitive to the usual oxidants, the method of choice to overcome sulfhydryl interference is to form a tight complex with a heavy metal such as Hg^{++}. It is 250 times as effective as Cu^{++} which has been used by Gibson *et al.* (11).

Balance Experiment—The PBG that was formed enzymically was equivalent quantitatively to the δAL that disappeared (Table II). This confirms the findings by Gibson *et al.* (11) for δAL-ase from beef liver.

According to the findings of Shemin (18) and of Berlin *et al.* (19), δAL is in part converted to other products than PBG. In the present experiments with enzymes from rabbit reticulocytes, δAL was found to be transformed only by those fractions which contained δAL-ase.

TABLE II

Quantitative Conversion of δAL to PBG by Rabbit δAL-ase

Time	δAL remaining	PBG formed	Decrease in moles δAL / Moles PBG formed	
			Uncorrected	Corrected
hrs.	*μmole*	*μmole*		
0	0.263			
1	0.133	0.0624	2.02	2.08
	0.135	0.0630	1.95	2.03
2	0.0821	0.090	1.95	2.01
	0.0821	0.090	1.95	2.01
3	0.0452	0.104	2.09	2.09
	0.0492	0.106	2.02	2.02
4	0.0274	0.120	1.90	1.96
	0.0260	0.114	2.06	2.08

After protein precipitation PBG was determined by an elaboration of the method described above, and δAL by the method of Mauzerall and Granick (16). Corrections (last column) were made for the spontaneous decrease during a 4 hour incubation period of δAL (9 per cent) and of PBG (10 per cent) and for the color contribution of the PBG to the δAL determinations (equivalent to 23 per cent of the PBG).

Stability of δAL-ase—Solutions of chicken or rabbit δAL-ase, preactivated in 0.01 M GSH, and containing more than 0.4 mg. of protein per ml., did not lose activity as rapidly as less concentrated solutions (Fig. 4). The maintenance of activity appears to be due to a stabilization of both the enzyme and of the PBG formed. PBG (5×10^{-4} M) incubated at 38° in PO_4 buffer, pH 7, for 4 hours was decomposed 10 per cent. The activity for short incubation periods was found to be directly proportional to the enzyme concentration over a 10-fold change in concentration of enzyme (Fig. 5). The pH dependence of the rabbit reticulocyte δAL-ase and of the chicken erythrocyte δAL-ase is shown in Fig. 6, *A* and *B*. The maximum is at pH 6.3 for the rabbit enzyme and at pH 6.7 for the chicken enzyme. A similarly shaped curve has been observed in the case of beef liver δAL-ase with a maximum at pH 6.7 (11). The rather marked decrease in δAL-ase activity on the acid side of the maximum is due in part to an irreversible inactivation occurring at acid pH at 38°. The half life of chicken δAL-ase at pH 5.3 and 38° is about 15 minutes, while that for the rabbit enzyme is about 1 hour. With the rabbit enzyme no loss in

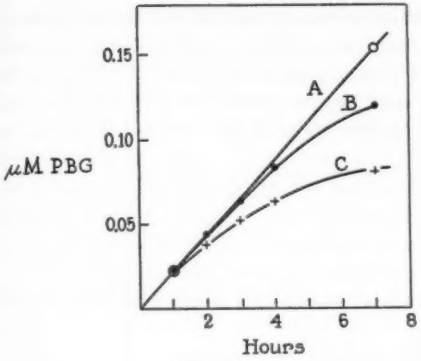


FIG. 4

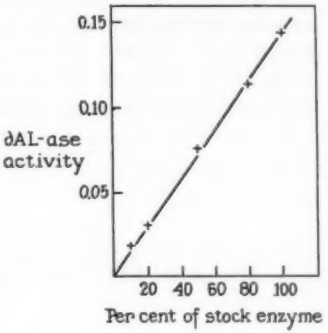


FIG. 5

FIG. 4. Stabilization of dilute δAL-ase by protein. Preactivated chicken δAL-ase (0.16 mg. of protein per ml.) was incubated in 0.02 M PO_4 buffer, pH 6.8, with 2.5×10^{-3} M δAL in the presence of (Curve A) 0.25 per cent bovine albumin, (Curve B) 1 per cent gelatin, (Curve C) no added protein. δAL-ase activity measured under standard conditions is in micromoles of PBG per ml. of enzyme per hour.

FIG. 5. δAL-ase activity *versus* enzyme dilution. Preactivated chicken δAL-ase stock solution (2.1 mg. of protein per ml.) in M/15 PO_4 buffer, pH 6.8, was diluted with the same buffer. Incubation time was 30 minutes, δAL concentration 2.5×10^{-3} M. δAL-ase activity is in micromoles of PBG per hour per ml. of diluted enzyme, measured under standard conditions.

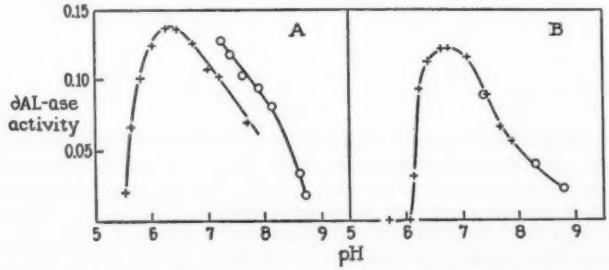


FIG. 6. δAL-ase activity *versus* pH of (*A*) preactivated rabbit reticulocyte preparation (0.32 mg. of protein per ml.); (*B*) preactivated chicken erythrocyte preparation (2.1 mg. of protein per ml.). δAL-ase activity is in micromoles of PBG per hour per ml. of enzyme measured under standard conditions. +, 0.05 M PO_4 buffer; ○, 0.05 M Tris buffer.

activity was found by treatment at 0° and pH 5.3 for 1 hour, nor at 38° and pH 7.9 for 1 hour. An inhibition by PO_4 buffer as compared to Tris buffer was observed with the δAL-ase of the rabbit (Fig. 6, *A*) but not with that of the chicken.

Substrates for δAL-ase—For the beef liver enzyme neither amino acetone, ε-amino-δ-ketohexanoic, nor α,δ-diamino-γ-ketopentanoic acid are substrates (11). When mixtures of amino acetone and δAL were incubated with rabbit δAL-ase, no mixed condensation products could be detected by paper electrophoresis. The methyl ester of δAL formed a pyrrole at

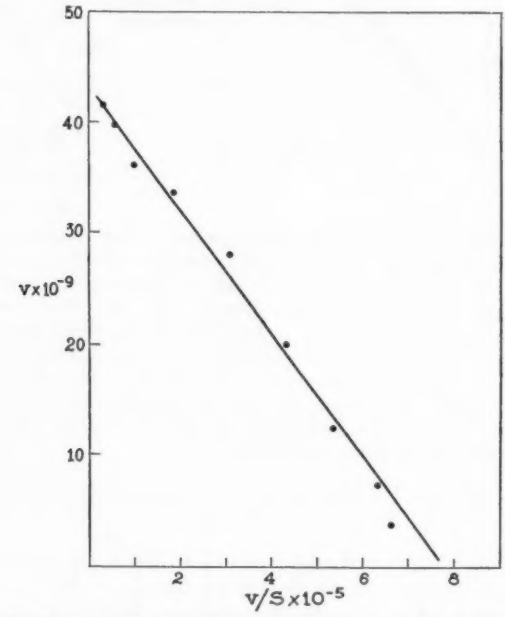


FIG. 7. Michaelis constant determination of preactivated rabbit δAL-ase (0.6 mg. of protein per ml.). v is plotted *versus* v/S, where v = the observed rate of PBG formation per hour per ml. of enzyme, and S = the molar concentration of δAL. The activity was measured under standard conditions. The points at low substrate concentration have been corrected to initial rates by assuming a first order rate law. The corrections ranged from 3 per cent at 2×10^{-3} M δAL to 15 per cent at 6×10^{-5} M δAL.

about one-half the rate of δAL. However, the ester is rapidly hydrolyzed and no mono- or diester of PBG could be found by paper electrophoresis.

Michaelis Constant of δAL-ase—The apparent K_m of both rabbit reticulocyte and chicken erythrocyte δAL-ase is 5×10^{-4} M (Fig. 7). Beef liver δAL-ase is reported to have a K_m of 1.6×10^{-4} M (11). All of these enzymes follow simple Michaelis-Menten type kinetics.

Inhibitors of δAL-ase—In confirmation of the findings of Gibson *et al.* (11), metals inhibit δAL-ase roughly in the order of the solubility products

of their sulfides. Iodoacetamide at 10^{-3} M inhibited the enzyme completely. These results and the activation by GSH indicate the presence of essential SH groups of this enzyme. With rabbit δAL-ase, the following compounds showed little or no inhibition below 10^{-1} M: butyrate, valerate, caproate, β-alanine, γ-aminobutyrate, δ-aminovalerate, ε-aminocaproate, and ethyl levulinate. At 10^{-2} M, pyruvate and acetoacetate were weakly inhibitory, and α-ketoglutarate and oxalacetate were more strongly inhibitory (Table III). At 10^{-3} M, only levulinate, succinic semialdehyde, and *p*-aminobenzoate were appreciably inhibitory.

The inhibition and substrate studies suggest that neither the α-amino ketone grouping alone nor an amino group, even if δ to the carboxyl, is

TABLE III

Inhibitors of Rabbit δAL-ase

1.1 mg. of protein per ml. The substrate was 2.5×10^{-3} M δAL. Other conditions were standard.

Inhibitor	Molarity at 50 per cent inhibition $\times 10^3$
Levulinate	3
Succinic semialdehyde	1
α-Ketoglutarate	10
Oxalacetate	20
Benzoate	8
p-Aminobenzoate	3
Naphthalene-1-amino-4-sulfonate	10
Sulfanilate	50
Sulfanilamide	20

sufficient to bind the δAL to the enzyme. The only moderately effective inhibitors are levulinate and succinic semialdehyde which have a keto group δ to an ionized carboxyl group.

EDTA Inhibition of δAL-ase—According to Gibson *et al.* (11), beef liver δAL-ase is markedly inhibited by EDTA, the activity decreasing sharply over a 4-fold change in EDTA concentration. With rabbit δAL-ase, the inhibiting effect of EDTA suggests more than one type of inhibition (Fig. 8). The slope of the activity curve below 50 per cent activity is less than expected for a simple 1:1 (enzyme to inhibitor) reversible inhibition. This simplest type of enzymic inhibition has been elegantly discussed in the classical papers of Strauss and Goldstein (20) and Goldstein (21). The inhibition curve was unaffected by an 8-fold increase in δAL concentration and is of the "uncompetitive" type (Fig. 9). The slope does not change but the intercept increases by $(1 + I/K_i)$ with $K_i = 4 \times 10^{-5}$ M (22). This type of inhibition is usually accounted for by the as-

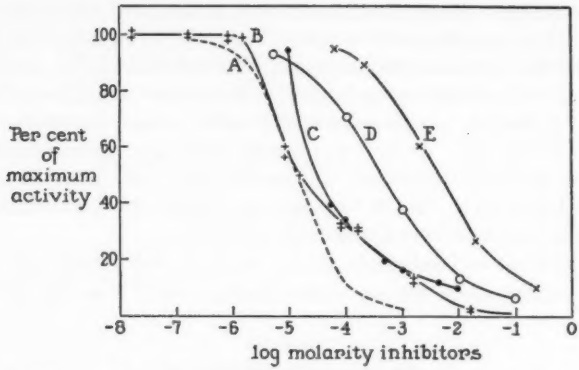


FIG. 8. Inhibitory action on preactivated rabbit δAL-ase (1.1 mg. of protein per ml.) of EDTA and related compounds. Incubation 2 hours, δAL 2.5×10^{-3} M; other conditions were standard. Curve A, the dashed line represents a theoretical curve for a simple reversible 1:1 (enzyme-inhibitor) inhibition; Curve B, EDTA; Curve C, CaNaEDTA; Curve D, $N(CH_2COOH)_3$; Curve E, $CH_3N(CH_2COOH)_2$.

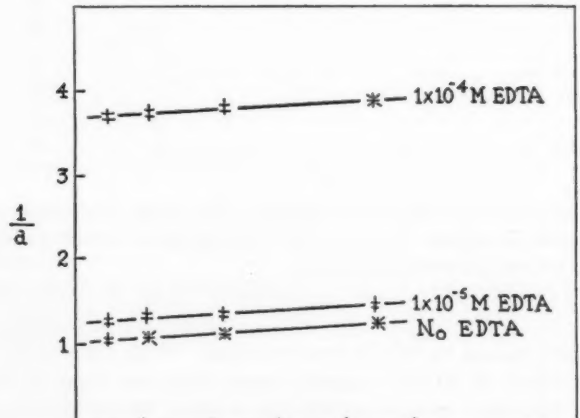


FIG. 9. Uncompetitive inhibition of preactivated rabbit reticulocyte δAL-ase (1.1 mg. of protein per ml.) by EDTA. Incubation 2 hours; δAL 2.5×10^{-3} M; other conditions were standard. Plotted as 1/density of PBG-Ehrlich color *versus* 1/molarity of δAL at 0, 1×10^{-4} M and 1×10^{-5} M EDTA concentrations.

sumption that the inhibitor, I, combines only with the enzyme-substrate complex. Although this enzyme acts on two identical substrate molecules, the kinetics found are of the simple 1:1 (enzyme-substrate) form (see under "Discussion") and therefore the general equations developed for enzyme inhibition by Segal *et al.* (22) are applicable. Similar results, however, would be obtained if EDTA removed an essential metal from the enzyme-substrate complex. The inhibition with EDTA may be par-

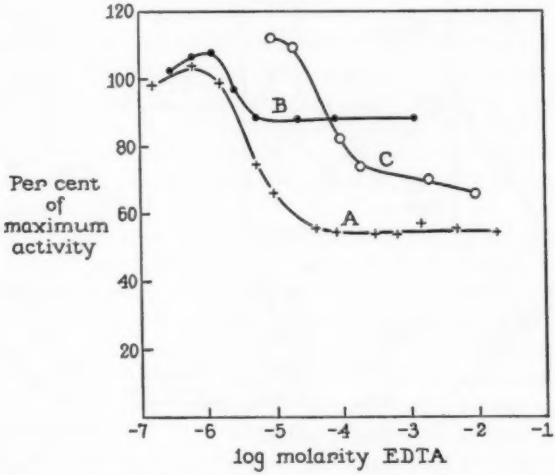


FIG. 10. Inhibition by EDTA of different preactivated chicken δAL-ase preparations, measured under standard conditions. Preparation A, from zone electrophoresis on starch. Enzyme concentration 0.6 mg. of protein per ml.; Preparation B, from zone electrophoresis on starch which was prewashed with bovine albumin. Enzyme concentration 1.1 mg. of protein per ml.; Preparation C, from bulk method. Enzyme concentration 1.2 mg. of protein per ml.

tially reversed. Overnight dialysis against dilute buffer resulted in a recovery of 56 per cent and in the presence of 0.01 M Mg^{++}, of 85 per cent of the activity. The inhibition by CaNaEDTA also does not follow a simple 1:1 reversible inhibition curve. However, the inhibitions by $N(CH_2COOH)_3$ ($K_i = 8 \times 10^{-4}$ M) and $CH_3N(CH_2COOH)_2$ ($K_i = 8 \times 10^{-3}$ M) do follow a simple 1:1 inhibition curve.

The complex nature of the inhibition by EDTA is revealed more clearly with the chicken enzyme. The inhibition depends on the preparation of the chicken δAL-ase. Only partial inhibition of any preparation was observed even at 10^{-2} M EDTA (Fig. 10). Preparation B, an eluate from zone electrophoresis on starch prewashed with bovine albumin, was only

slightly inhibited by EDTA. The slight increase of activity above 100 per cent at very low EDTA concentration may be due to the removal of inhibiting trace metals not removed by GSH. Preparation C, made by the bulk method, contains a brown iron protein which migrates electrophoretically like ferritin; higher concentrations of EDTA ($>10^{-5}$ M) are seen to be required for inhibition.

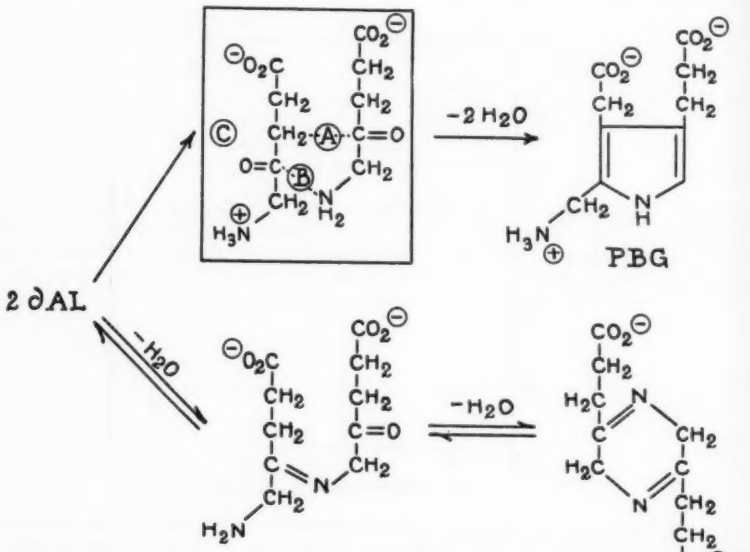


FIG. 11. Mechanism of action of δAL-ase

Properties of δAL—In alkaline solution δAL condenses to a ketimine (Fig. 11) and dihydropyrazine (10). When δAL is kept at pH 6.8 and 40° for 2 hours, less than 10 per cent is in the form of a ketimine or dihydropyrazine. On paper electrophoresis at pH 6.8, 40°, δAL migrates as a dipolar ion. However, at pH 8.0, 40°, material moving faster towards the anode than δAL is observed, indicating that appreciable condensation to ketimine or dihydropyrazine takes place. Titration of δAL to pH 10 at 22°, acidifying, and retitration within 1 hour give duplicate curves. The pK values of δAL are 4.0 and 8.2 ± 0.1. Under the conditions used for the determination of δAL-ase activity the non-enzymic condensation

of δAL to Ehrlich-positive materials is negligible at concentrations below 5×10^{-2} M.

Mechanism of δAL-ase Action—The stoichiometry of the condensation of 2 molecules of δAL to form 1 PBG (Fig. 11) has been established by both chemical (Table II), (11) and labeling (3) methods. The condensation requires the formation of a bond at *A* and at *B*. The following arguments suggest that only one enzyme is involved in PBG formation. (*a*) Only one, fairly symmetrical zone of δAL-ase activity was found on zone electrophoresis of the supernatant fluid from the red cells of the three species studied. The activity of the eluate of this zone was not enhanced by combination with eluates from other zones. (*b*) The enzyme-substrate kinetics are of the simple 1:1 type (Fig. 7). (*c*) The activity *versus* enzyme dilution is linear (Fig. 5). The latter two arguments, however, could be vitiated by a series of enzymes which rapidly come to steady state conditions. Following the usual simplicity principle, we shall refer to δAL-ase as one enzyme.

In addition to the usual forms of enzyme inactivation noted above, the inhibition by EDTA may indicate another kind of change. The usual *ad hoc* hypothesis to explain the incomplete inhibition as observed in Fig. 10 is that the enzyme-substrate-inhibitor complex reacts at a slower rate than the enzyme-substrate complex; however, this would not explain the variability encountered. Another hypothesis is that two different proteins with δAL-ase activity are present in variable proportions, only one of which is inhibited by EDTA. Attempted separation of these proteins by zone electrophoresis was unsuccessful; when the zone of δAL-ase activity was divided into a slow, middle, and fast portion, the EDTA inhibition curves were the same for the three portions. Possibly handling causes modifications of some of the δAL-ase molecules so that the modified molecules become susceptible to inhibition by EDTA.

The inhibition by EDTA may be due either to a competition for an essential metal or to direct binding on the enzyme. The fact that with rabbit δAL-ase the 50 per cent point of inhibition by EDTA, $N(CH_2COOH)_3$, and $CH_3N(CH_2COOH)_2$ (Fig. 8) is in the order of their metal-chelating abilities (*e.g.* for Mg^{++}) is suggestive of an inhibition due to competition for an essential metal.

Two possible general schemes for δAL-ase action may be considered, depending on whether δAL or the ketimine is the immediate substrate.

(A) $$2S \rightleftharpoons S_2 \qquad E + S_2 \rightleftharpoons ES_2 \rightarrow E + P$$

(B) $$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad ES + S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES_2 \xrightarrow{k_5} E + P$$

Scheme A assumes a preliminary non-enzymic condensation to a dimer,

S_2, the ketimine. This condensation cannot be rate-determining for the rate is proportional to the enzyme concentration (Fig. 5). If the spontaneous condensation were very rapid and if very little S_2 were formed, the rate would follow an enzyme-substrate plot second order in substrate concentration. Actually a plot of v *versus* v/S_2 is highly curved, eliminating this possibility. If the spontaneous condensation to S_2 were very rapid and if most of S were in the form of S_2, then the results obtained could be explained. However, less than 10 per cent of the δAL was found to be in the form of the ketimine at pH 6.8 so that Scheme A may be rejected.

In Scheme B the formation of ES_2 cannot be rate-determining, for then the kinetics would be second order in substrate concentration. If the formation of ES were rate-determining, the rate would be continually proportional to S, whereas it actually tends to a limit. If we assume the formation of P to be the slow step, *i.e.* that the binding is at equilibrium, then the dissociation of ES must be less than that of ES_2 in order to explain the observed kinetics. In this respect they resemble certain enzyme-coenzyme-substrate reactions. A more general approach is obtained by applying the steady state conditions to Scheme B. The following equation for the initial rate of product formation is obtained

$$v = \frac{k_5 E_0}{1 + \left[\frac{k_4 + k_5}{k_3} + \frac{k_5}{k_1}\right]\frac{1}{S_0} + \frac{k_2}{k_1}\left[\frac{k_4 + k_5}{k_3}\right]\frac{1}{S_0^2}}$$

The condition required for the term in $1/S_0^2$ to be negligible is that

$$S_0 > k_2/[k_1 + k_3k_5/(k_4 + k_5)]$$

Again if k_5 is small, the dissociation of ES, k_2/k_1, must be below 10^{-4} M, the lower limit of S_0 used in these experiments. Elimination of other possibilities and more definite proof of this hypothesis would require measurements at still lower concentrations and preferably in the non-steady state. The suggestion that the enzyme has two sites for the δAL molecules is consistent with the fact that "mixed" pyrroles were not observed with δAL and amino acetone or δAL methyl ester, *i.e.* both sites are fairly specific.

The condensation of δAL to PBG resembles a Knorr pyrrole condensation. The inhibition data suggest that a carbonyl group γ to an ionized carboxyl group is necessary for binding to the active site of δAL-ase (Fig. 11). Kinetic data suggest but do not prove the following mechanism for the action of this enzyme: Both molecules of δAL form complexes with δAL-ase, the first being held more tightly than the second. The binding of this second δAL molecule may involve spontaneous formation of the ketimine at B. The presence of a metal ion at C would favor the forma-

tion of an enolate ion. The aldol condensation requires an enolate ion to attack the carbonyl of the adjacent δAL at *A*. This condensation at *A* with hydrogen shift would then result in pyrrole formation.

PBG-ase

Measurement of PBG-ase Activity—PBG-ase activity is defined as the micromoles of PBG which react in 1 hour per ml. of enzyme solution. Standard conditions for measurement of the activity are the following. To 0.2 ml. of enzyme solution are added 0.1 ml. of M/15 PO_4 buffer, pH 7.3, and 0.1 ml. of 2×10^{-3} M PBG. The mixture is incubated aerobically at

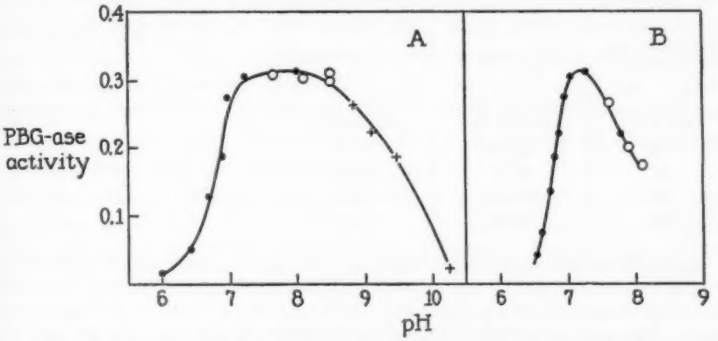


FIG. 12. PBG-ase activity *versus* pH. *A*, rabbit reticulocyte enzyme (0.35 mg. of protein per ml.); *B*, chicken erythrocyte enzyme (2.1 mg. of protein per ml.). ●, M/15 PO_4 buffer; ○, 0.1 M Tris buffer; +, 0.1 M glycine buffer. The enzymes were predialyzed against water. Incubation, 2 hours at 38°; other conditions were standard.

38° for 1 to 2 hours in the dark. Blanks without enzyme are run simultaneously. Dilution of the enzyme is so chosen that about 25 per cent of the PBG is converted. After incubation and precipitation of the proteins, the PBG remaining is determined colorimetrically (see "Measurement of δAL-ase activity").

Some Properties of PBG-ase—The pH-activity curves for preparations from rabbit and chicken red cells are presented in Fig. 12. The curves obtained are probably composites resulting from the activities of more than one enzyme (see below). Some inhibitors of PBG-ase were studied. No inhibition was found with EDTA (2×10^{-3} M) or with iodoacetamide and *N*-methyl maleimide (2×10^{-4} M). GSH (10^{-2} M) inhibited the activity 30 per cent. Cu^{++} (2×10^{-4} M), Hg^{++} and *p*-chloromercuribenzoate (5×10^{-6} M), inhibited the activity 50 per cent.

Conversion of PBG to URO'gen III by PBG-ase—The product of action

of PBG-ase on PBG under anaerobic conditions is very likely URO'gen, as indicated by the appearance of porphyrin bands only following oxidation of the reaction mixture with iodine. Under the aerobic conditions used, a large portion is autoxidized to the porphyrin and a further portion is autoxidized to a product which has an intense band at 500 mμ below pH 8.5. This intermediate and the URO'gen may be oxidized to URO with iodine (23). The porphyrin formed was identified as URO by paper chro-

TABLE IV

Effect of Heat Treatment of Rabbit PBG-ase on URO Isomer Produced

Preliminary heat treatment	Total porphyrin	Porphyrin formed	Relative intensity of ester spots by Falk and Benson method	
			URO type III	URO type I
min. at 55°	*moles × 10⁹*	*per cent theory*		
0	41.0	80.4	+++	0
15	42.8	84.0	+±	++
30	42.9	84.2	+±	++
60	40.0	78.4	+	+++

Each tube containing 0.2 ml. of enzyme (0.35 mg. of protein per ml.) plus 0.1 ml. of M/15 PO_4 buffer, pH 7.3, was heated at 55° for the designated period. The tubes were then incubated aerobically at 38° with 0.1 ml. of PBG (205×10^{-9} mole) for 15 hours. The porphyrins were extracted by the addition of 0.5 ml. of 5 per cent TCA; the residue was centrifuged and reextracted with 1 ml. of 1 N HCl. The combined aqueous porphyrin extract was washed with ether to remove TCA. The density was determined at 548 mμ and the yield of porphyrin was calculated from $\epsilon = 1.7 \times 10^4$. The solutions were then evaporated to dryness, esterified with methanol-HCl, and the type of the URO isomer was determined by the Falk and Benson method (25).

matography according to Nicholas and Rimington (24) and as the type III (or IV) isomer by the Falk and Benson method (25).

Deaminase and Isomerase Activities—It was found by Bogorad and Granick (7) that *Chlorella* extracts exposed to 55° for 30 to 60 minutes formed COPRO I from PBG, whereas unheated extracts formed COPRO III. With rabbit PBG-ase such treatment results in a decrease in URO III and a simultaneous increase in URO I, without affecting the total yield of porphyrin (Table IV). Booij and Rimington (26) have noted a similar isomer change with heated red cells.

The effect of heat treatment suggests that the PBG-ase preparation has more than one action. It seems likely that one is a "deaminase" action which condenses PBG molecules to form polypyrrylmethanes by elimination of NH_3; another is an "isomerase" action which by an unknown mech-

anism inverts 1 (or 3) of the PBG molecules to give an isomer III sequence. The isomerase action is then the one that would be readily destroyed by heat. Bogorad (27) has succeeded in separating two activities from plant extracts and has shown that the deaminase action occurs prior to the isomerase action.

To test whether the isomerase might act on URO'gen I, this compound (23) was incubated with an active rabbit PBG-ase preparation that would normally convert PBG to URO'gen III. No URO III was found after oxidation. Thus the isomerase must act at a stage preceding the condensation of some intermediate to the porphyrinogen ring.

PBG has been found to be converted to the extent of 90 ± 10 per cent to URO III by PBG-ase. If the mechanism of the isomerase action is by transfer of the $—CH_2—NH_2$ group as hypothesized for the chemical condensation by Cookson and Rimington (13), this result with the enzyme suggests that no haphazard loss of HCHO or $HCHO—NH_2$ occurs into solution, but rather that the transfer of this group occurs via the enzyme or suitable coenzyme, *e.g.* tetrahydrofolic acid.

In the disease porphyria congenita, which is due to a recessive gene, large amounts of URO I and COPRO I are excreted (28). The available data suggest that one of the effects of this disease is a diminution in active "isomerase" enzyme of the bone marrow.

Uroporphyrinogen Decarboxylase

Observations on this enzyme are given in the following paper (23) in which proof is presented that the substrate is the fully reduced porphyrin, *i.e.* uroporphyrinogen.

DISCUSSION

The isolation of three soluble enzyme fractions from erythrocytes has made possible the examination of several individual steps in porphyrin biosynthesis. The results may be interpreted and summarized according to the scheme of Fig. 13.

2 molecules of δAL are condensed to PBG by δAL-ase by a mechanism such as discussed earlier. The condensation of PBG to URO'gen III seems to require two kinds of enzymic reactions. 2 to 4 molecules of PBG are condensed by a "deaminase" to give a hypothetical polypyrrylmethane and ammonia. Continued action of this enzyme leads to URO'gen I. However, by the intervention of the "isomerase" reaction, 1 (or 3) of the PBG residues is inverted by an unknown mechanism leading on ring closure to URO'gen III. Both URO'gen I and III are acted upon by the decarboxylase, UD-ase, which successively removes carboxyl groups from the acetic acid side chains to give COPRO'gen I or III. COPRO'gen III

(but not I) is oxidatively decarboxylated by an enzyme bound to insol components of the cell to form (after autoxidation) protoporphyrin (

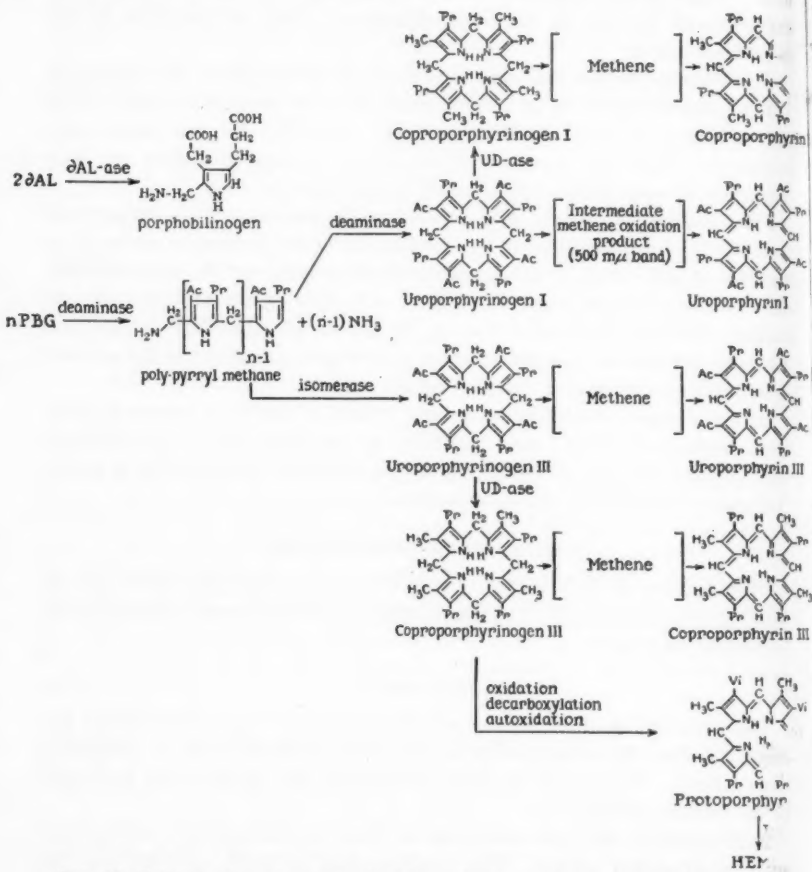


FIG. 13. Scheme of protoporphyrin biosynthesis in the red cell from δ-ami levulinate to iron protoporphyrin. Ac = CH_2COOH, Pr = CH_2CH_2COOH, Vi $CH{=}CH_2$, δAL-ase = enzyme that condenses δ-aminolevulinic acid to porphobili gen, UD-ase = enzyme that decarboxylates uroporphyrinogen to coproporphyri gen.

Whether the iron is inserted at an intermediate stage of autoxidation PROTO or into PROTO itself is unknown. However, under conditio whereby PROTO is not colloidal at neutral pH and 38°, ferrous iron w coordinate to form heme in th absence of enzymes.

Concurrently with the above enzyme reactions any of the porphyrinogens may be autoxidized, through pyrrylmethene-like intermediates which have a characteristic absorption at 500 mμ, to the respective porphyrins which themselves are inactive. These autoxidations are photocatalyzed by the porphyrins formed and are therefore autocatalytic (23) in the presence of light. The amount of these porphyrin byproducts formed (mostly URO and COPRO) depends not only on the activities of the various enzymes involved but also on the presence of catalysts (light) or inhibitors (GSH) of their autoxidation.

We acknowledge our gratitude to Mrs. Annabelle Long and Mr. William Cumming for able technical assistance, to Dr. R. J. Slater for a sample of erythroblastosis fetalis blood, to Dr. Henry Kunkel for advice on zone electrophoresis methods, to Dr. J. Dice for his procedure of δAL synthesis, to Professor C. Rimington for samples of URO and COPRO isomers, to Dr. S. F. MacDonald for synthetic samples of URO isomers, and to Dr. A. Bearn for the suggestion of albumin treatment of starch to trap heavy metals.

SUMMARY

Three soluble enzyme fractions involved in porphyrin biosynthesis have been obtained from red cells of chicken, rabbit, and man by zone electrophoresis. One fraction condenses δ-aminolevulinic acid to porphobilinogen, another converts porphobilinogen to uroporphyrinogen III, and a third decarboxylates uroporphyrinogen to coproporphyrinogen.

Kinetic and inhibitor studies on the first fraction show that the enzyme specifically binds both molecules of δ-aminolevulinic acid required for the condensation to porphobilinogen. Studies of the uroporphyrin isomers produced by the second fraction indicate that two types of reactions are involved. General mechanisms for the above reactions are discussed.

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PORPHYRIN BIOSYNTHESIS IN ERYTHROCYTES

III. UROPORPHYRINOGEN AND ITS DECARBOXYLASE*

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(Received for publication, August 21, 1957)

The porphyrinogens are colorless, reduced porphyrins containing 6 extra atoms of hydrogen. They have been reported to occur in biological materials by Fischer, who isolated COPRO'gen[1] from the feces of his porphyria patient Petry (1), and by Watson, Schwartz, and coworkers, who showed that COPRO'gen was present in the urine of some of their patients with various porphyrias (2). Suspicions that these porphyrinogens are the actual intermediates in the biosynthesis of porphyrins arose when it was observed that the porphyrins themselves, *i.e.* URO and COPRO, could not be used for heme or chlorophyll synthesis by either intact or disrupted cells (3–5). The structure of porphobilinogen gave further indirect evidence for this view since a condensation of 4 molecules of this pyrrole would produce URO'gen (6). Also, Bogorad obtained small amounts of COPRO on incubating reduced URO with a preparation from *Chlorella* (5), and Neve, Labbe, and Aldrich have found that adding reduced URO to hemolyzed red cells increased the incorporation of Fe^{59} into heme (7).

We have isolated by zone electrophoresis on starch an enzyme preparation from red cells which decarboxylates uroporphyrinogen but not uroporphyrin, yielding coproporphyrinogen as the final product. Some observations have been made on the photocatalytic autoxidation of URO'gen, and the structure of compounds at levels of reduction intermediate between URO and URO'gen is discussed.

EXPERIMENTAL

Materials

Porphyrins—Unless otherwise mentioned, the experiments reported here were made with a URO octamethyl ester mixture, m.p. 260°, synthesized by

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, No. R.G.-4922.

[1] Abbreviations used are as follows: URO'gen = uroporphyrinogen; URO = uroporphyrin; COPRO'gen = coproporphyrinogen; COPRO = coproporphyrin; GSH = glutathione; EDTA = ethylenediaminetetraacetic acid; Tris = tris(hydroxymethyl)aminomethane; mμmoles = millimicromoles = 10^{-9} mole.

Dr. S. F. MacDonald (8). Isomers I and II had been removed by repeated crystallization; paper chromatography by the Falk-Benson method (9) revealed a single porphyrin ester with the mobility of pure URO III. A few early experiments were carried out with a sample of URO obtained by the chemical condensation of porphobilinogen. This was characterized as URO III by the same criteria. URO octamethyl esters I, II, and IV were synthetic samples prepared by Dr. S. F. MacDonald and coworkers (10, 11). COPRO III tetramethyl ester, m.p. 162°, remelted, 176°, was obtained from a fraction of broth used in the preparation of diphtheria toxin, through the courtesy of Dr. F. H. Clarke of the Lederle Laboratories. Authentic samples of URO III octamethyl ester and COPRO I tetramethyl ester were obtained from Dr. C. Rimington.

Small samples of the porphyrin esters were hydrolyzed with 6 M HCl for 1 day at 25° in the dark. The acid was evaporated *in vacuo* and the porphyrin dissolved in the required amount of KOH solution. This stock solution of $\sim 10^{-3}$ M was kept frozen until used. 2,2′,4,4′-Tetramethyldipyrrylmethene was obtained from Dr. A. Corwin. In later experiments the distilled water was passed through a deionizing column, but no significant changes in enzyme activity or porphyrin recoveries were observed. Ether was freshly distilled and free from peroxides. Other chemicals were of reagent grade.

Enzyme—The preparation of the enzyme by zone electrophoresis on starch of the soluble components of hemolyzed rabbit red cells has been described in a preceding paper (12). The solutions contained ~0.1 mg. of N per ml.

Methods—In all operations with the porphyrins, exposure to light was minimized. Spectrophotometer readings were made at room temperature with a Beckman model DU spectrophotometer, equipped with a diaphragm near the light exit and with a modified cell holder which permitted the assay of 1 ml. samples. Spectra were usually measured on a Cary recording spectrophotometer, model 11. Since the porphyrin bands are relatively narrow, minimal slit widths must be used. In later experiments a photo multiplier was added to the Beckman apparatus, and a slit width of ~0.01 mm. was used from 400 to 650 mμ. A hand spectroscope was invaluable for preliminary experiments and as a control on extractions, photooxidations, etc.

Uroporphyrinogen Preparation—Under dim red light about 2 gm. of freshly ground 3 per cent sodium amalgam are added to about 4 ml. of a solution of URO ($\sim 4 \times 10^{-4}$ M) in very dilute KOH. The flask is flushed with N_2, stoppered, and shaken vigorously. The fluorescence disappears within 3 minutes. The solution is rapidly filtered through a fine sintered glass disk with suction and titrated under nitrogen to pH 6.8 with 40 per

cent H_3PO_4. The resulting solutions were used immediately, but can be kept in the dark, and still better at dry ice temperatures. Coproporphyrin was reduced in the same manner.

Extraction of Porphyrinogens—URO'gen, unlike URO, is readily extracted by ethyl acetate at pH 3. At this pH neither URO, URO'gen, nor the partially oxidized porphyrinogens is extracted by ether. When a mixture of URO'gen and enzyme that had been incubated anaerobically was rapidly extracted with ether at pH 3, it was possible to remove only the COPRO'gen (and COPRO forming during the extraction). By reextraction of these compounds into a buffer solution and spectrophotometric titration with I_2

TABLE I

Extinction Coefficients for URO and COPRO Porphyrins

	URO		COPRO	
	mμ	$\epsilon \times 10^{-4}$	mμ	$\epsilon \times 10^{-4}$
HCl, 1 M	407	50	402	47
" 1 "	553	1.7	549	1.7
" 1 "	594	0.57	592	0.61
Sodium phosphate, pH 6.8, 0.067 M	503	1.3	505	0.53
Sodium phosphate, pH 6.8, 0.067 M	539	1.1	538	0.54
Sodium phosphate, pH 6.8, 0.067 M	561	0.87	562	0.52
Sodium phosphate, pH 6.8, 0.067 M	613	0.40	609	0.23

ϵ = (1/cm. × mole per liter) $\log_{10} I_0/I$, slit widths ~ 0.01 mm.

the amount of both COPRO and COPRO'gen could be determined, and the reduction level of the COPRO'gen estimated.

Analysis—Since the spectra of these porphyrins vary in a complex way with pH and ionic strength near neutrality (13), all measurements were made in acid solution, usually 1 M HCl, where these effects are much smaller. The extinction coefficients given in Table I are in agreement with those found in the literature (13–15). Beer's law was closely followed in acid solution for concentrations up to at least 7×10^{-5} M. At pH 6.8 only small deviations in the extinction coefficients occur with URO. Solutions of COPRO (2 to 20×10^{-5} M) which are partly colloidal at this pH show a decrease of 10 per cent.

The following standard procedure was adopted for incubation and analysis. Larger experiments were scaled up accordingly. 0.2 ml. of 0.067 M phosphate buffer, pH 6.8, plus additives (GSH, inhibitor, etc.) was added

to 0.5 ml. of enzyme solution (at pH 6.8) in a small test tube. Under dim red light 0.1 ml. of URO'gen ($\sim 3 \times 10^{-4}$ M) at pH 6.8 was mixed in, and the tubes were placed in brown bottles containing pyrogallol and vials of a concentrated NaOH solution. The bottles were closed with stoppers containing inlet and outlet tubes, and "prepurified" N_2 was rapidly passed through for 15 minutes. The tubes were clamped off, the vials of NaOH solution spilled on the pyrogallol, and the bottles kept at 38° for the required time, usually 4 hours. On removal, the enzymic solutions were rapidly titrated with I_2, a hand spectroscope being used to follow the reaction. The appearance of the porphyrin bands at 540, 560, and 610 mμ and the rather sudden sharpening of the 500 mμ band gave a clear end point; any excess I_2 was reduced with thiosulfate. The pH was lowered to 3.5 by adding 1 M formic acid. The solution was extracted three times with 2 ml. portions of ether. The ether extract was washed with 2 ml. of 0.1 M formate buffer, pH 3.5, 2 ml. of H_2O, and then extracted twice with 1 ml. of 1 M HCl. The volume of the extract in graduated test tubes was noted, and the optical density at 402 mμ (or 549) was determined. The total millimicromoles of porphyrin present was calculated from the extinction coefficients of Table I. Control experiments without enzyme gave negligible readings (<1 per cent). The porphyrin formed was mostly COPRO with some porphyrins containing five and six carboxyl groups, as established by paper chromatography with 2,6-lutidine-water (16). URO could be assayed by making the aqueous phase 1 to 2 M in HCl, centrifuging, and determining the optical density at 553 or 407 mμ. The conditions described were sufficiently anaerobic to keep >80 per cent of the URO'gen reduced for 4 hours at 38°.

In control experiments with mixtures of URO (0 to 1.2×10^{-4} M) and COPRO (0.1 to 4×10^{-5} M) in the protein solution, about 80 per cent of the COPRO was recovered. At higher concentrations of URO, less COPRO was recovered, very likely due to coprecipitation. The URO concentration was therefore kept below this limit. The recovery of URO was 95 per cent. Since recovery of the URO'gen after standing for 4 hours at 38° under N_2 was only about 75 per cent, control experiments were included in each run. This allowed a reproducibility of ±5 per cent to be attained.

Isomers—The URO ester isomers were estimated by the Falk-Benson method (9) which distinguishes isomers I and II from III and IV, but not from each other. The total amount of porphyrin placed on the paper is highly critical, about 0.3 γ being optimal for Whatman No. 1. When the amounts of porphyrin are smaller, isomer I tends to have the same mobility as isomer III and with too much porphyrin isomer III lags behind, thus resembling isomer I (17). About one-eighth of isomer I (or II) in isomer III (or IV) could be determined.

The COPRO ester isomers were estimated by a modification of the method of Rappoport *et al.* (18). About 0.5 γ of unknown COPRO ester (freshly dissolved in $CHCl_3$) is placed on Whatman No. 1 paper along with the same amount of COPRO I and III esters and suitable mixtures of these isomers. Chromatography is done by the ascending method, on cylinders of the paper, the sides being held together by Teflon split rings. The esters are allowed to move 3 to 4 cm. from the base line with a solvent of 25 ml. of $CHCl_3$ and 30 ml. of petroleum ether (b.p. 60–70°). The paper is removed, dried, and cut 2 cm. below the spots. Separation of the isomers occurs with the second solvent system: petroleum ether:*tert*-butyl alcohol: 1,2-dichloroethane, 40:3:2 by volume. COPRO I ester remains at the origin while COPRO III ester moves near the solvent front. The composition of the second solvent may be changed to obtain maximal separation of the isomers with known samples. As in the Falk-Benson method for separating the URO ester isomers, the amount of porphyrin placed on the paper is critical. About one-eighth of isomer I in isomer III can be detected and *vice versa*. No known COPRO II and IV esters were available, but the COPRO esters isolated from the enzymic decarboxylation of URO'gen isomers II and IV showed a chromatographic behavior similar to that of isomers I and III respectively.

In these methods of separating URO and COPRO ester isomers the slower moving isomers (I and II) are markedly less fluorescent at the end of the separation than an equivalent amount of the faster moving isomers (III and IV). This observation and the critical dependence on amount of porphyrins suggest that both of the methods depend on fractional precipitation of the less soluble isomers onto the paper fibers. Spraying with 6 M HCl is useful to intensify the fluorescence of the slow moving isomers.

When the spectra of URO esters I and III in ether-alcohol were measured at liquid nitrogen temperature, an increase in intensity, 3 to 5 mμ shifts to shorter wave lengths, and a partial resolution of fine structure of the 500, 530, and 570 mμ bands were observed. However, the spectra of the two isomers were identical within experimental error.

Specificity of Enzyme Preparation—This enzyme preparation does not decarboxylate porphobilinogen or δ-aminolevulinic acid, as was determined by incubating portions of the enzyme with these compounds, then analyzing the mixtures by paper electrophoresis at pH 5.0. Only spots corresponding to the original material were found. Porphobilinogen was detected by spraying with Ehrlich's reagent. δ-Aminolevulinic acid was detected by spraying with 10 per cent acetylacetone at pH 7, incubating at 60° in a sealed jar for 0.5 hour, then spraying with Ehrlich's reagent.

2,2',4,4'-Tetramethyldipyrrylmethene; pK and Equilibrium with Sulfite Ion—The pK was determined by measuring the spectra of the compound

in the Cary spectrophotometer from pH 1.5 to 12.3. Phosphate, Tris, ammonia, and borate buffers (0.1 M) were used in the pH range 7 to 10. Dilute solutions of the compound are very photosensitive and therefore were used immediately after preparation. The amount of pseudo base present at alkaline pH with this alkyl dipyrrylmethene should be small (19).

The reaction with sulfite ion was measured at pH 5.3 (phthalate) and pH 7.0 (phosphate) by recording the density of the 463 mμ band of the protonated dipyrrylmethene. The ionic strength was 0.2 and the equilibrium constant for Reaction 1 (Fig. 4) was calculated with 7.1 as the second pK of sulfurous acid (20) and 8.4 as the pK of the dipyrrylmethene. All measurements were carried out at 22° ± 1°.

Photooxidation Experiments—The solution of URO'gen adjusted to the appropriate pH and containing in some cases inhibitors was placed in a Beckman cuvette and irradiated at the specified distance from the light source. A water heat filter was used in combination with a No. 2 photoflood lamp. Porphyrin formation was followed by measuring the intensity of the 560 mμ band, since the 500 mμ band was swamped and the 540 mμ band highly contaminated by the 500 mμ band of the intermediate oxidation products. The intensity of the 610 mμ band was used as a check on the 560 mμ band. The concentration of intermediates was determined by measuring the intensity at 500 mμ and subtracting the contribution from the porphyrin present. When oxidation was complete, the porphyrin concentration was determined by reading the 550 and 590 mμ bands in 1 M HCl. A reading at 500 mμ served to check the purity of the porphyrin, a high reading indicating the presence of decomposition products. Addition of I_2 at the end of the photooxidation gave no change in the spectra.

RESULTS AND DISCUSSION

URO'gen Preparation—Fischer prepared the porphyrinogens by a wide variety of methods (21). To these methods may be added that of slowly acidifying an alkaline solution of potassium borohydride and the porphyrin. However, shaking with freshly ground 3 per cent sodium amalgam in the dark under nitrogen was found to be the most specific, rapid, and convenient method. Under these conditions the absorption bands of the porphyrin fade out, and the fluorescence disappears shortly thereafter. In the light, or with old amalgam, the color of the solution changes from red through brown or green, decolorizing slowly, and showing a band at 640 mμ. On reoxidation such solutions never gave quantitative recovery of porphyrin, but showed much background absorption (at 500 and 640 mμ).

When samples of the URO'gen were oxidized with iodine at pH 7 and

assayed for porphyrin in acid solution, it was found that the reduction product contained 5.9 ± 0.1 hydrogen atoms and that 95 ± 4 per cent of the URO was recovered. Spectrophotometric or potentiometric titration gave the same result.

A solution of URO'gen showed a band at about 202 mμ, $\epsilon = 5 \times 10^4$, with a shoulder at about 220 mμ, $\epsilon = 3.5 \times 10^4$. This fact, together with the reduction to the hexahydro stage and the nearly quantitative recovery of

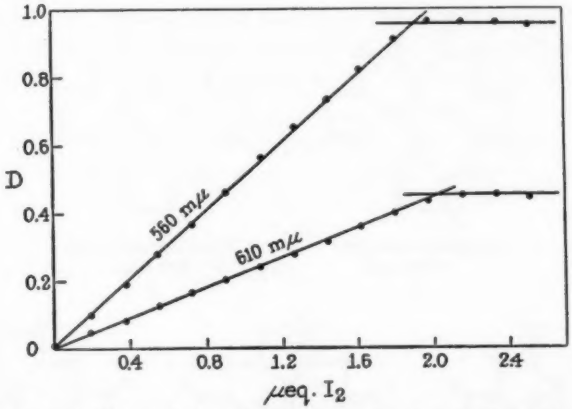


FIG. 1. A 3.47×10^{-4} M solution of URO was reduced and adjusted to pH 6.8 as described under "Experimental." 1.0 ml. of this solution and 2.0 ml. of M/15 phosphate buffer, pH 6.8, were added to a Beckman cuvette, and the mixture was titrated with 0.10 N I_2 with a calibrated micropipette. The resulting totally oxidized solution contained 0.329 μmole of URO by assay in acid solution, showing a recovery of 95 per cent. The end point of the titration is at 1.92 μeq. by the 560 mμ plot, indicating that the URO'gen contained 5.8 H atoms. The less reliable 610 mμ plot indicates 6.2 H atoms.

porphyrin on reoxidation, proves that the preparation contained at least 95 per cent URO'gen of Structure I, Fig. 2.

URO'gen Oxidation—At neutral pH and room temperature in the dark URO'gen is rapidly oxidized by iodine or lead peroxide and more slowly by certain quinones (*e.g.* tetrachloroquinone) and ferricyanide and dichromate ions. However, excess of lead peroxide or ferricyanide ion destroys the URO'gen. The reaction with iodine is instantaneous and quantitative and is the method of choice for oxidizing the URO'gen (2). The linearity of the plot of optical density at 560 or 610 mμ *versus* microequivalents of iodine added allows an accurate end point determination (Fig. 1). At acid pH, URO'gen is rapidly oxidized by ferric ion or chromium trioxide. Ceric ion destroys the URO'gen.

During all of these oxidations at pH <8, an intense absorption at 500 mμ appears and subsequently disappears completely, the final spectrum being that of a porphyrin alone. The photocatalytic autoxidation of the URO'gen will be discussed after considering the structure of this intermediate absorbing at 500 mμ.

Intermediate Absorbing at 500 mμ—The first stable molecule produced on oxidizing the colorless URO'gen (I), Fig. 2, would be the tetrahydropor-

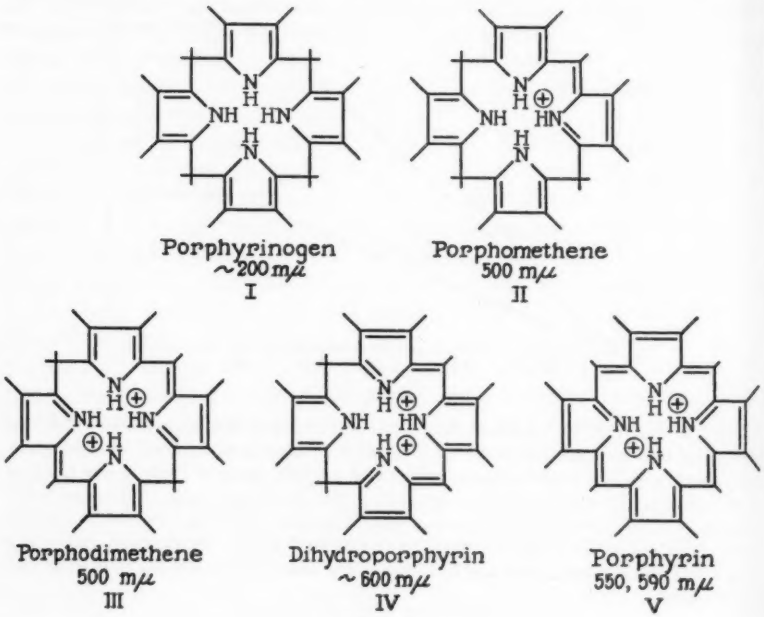


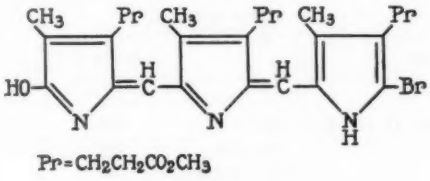
FIG. 2

phyrin (II). This is a *cis*-dipyrrylmethene. The corresponding noncyclic compounds absorb intensely at 460 to 490 mμ, *e.g.*: 2,2′,4,4′-tetramethyl-3,3′-diethyldipyrrylmethene perchlorate in dioxane, 485 mμ, $\epsilon = 8.3 \times 10^4$; free base, 442 mμ, $\epsilon = 2.1 \times 10^4$; zinc complex, 503 mμ, $\epsilon = 1.5 \times 10^5$ (22). Removal of 2 more hydrogen atoms presents two alternatives: attack on the opposite methane carbon leading to Structure III, or attack on the adjacent methane carbon leading to Structure IV. The expected spectrum for Structure III would be close to that of Structure II, but with about double the molar extinction coefficient. On the other hand Structure IV would absorb at longer wave lengths; the closest model is the tripyrrylene VI (Fig. 3), the zinc complex of which has a strong band at 627

mμ (23). Also, the hydrochloride of mesobiliviolin has an absorption maximum at 607 mμ, $\epsilon = 2.1 \times 10^4$.

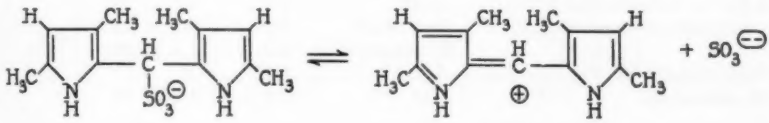
Structure III would be expected to be somewhat more stabilized by resonance than Structure IV, and therefore would predominate. On oxidation of URO'gen with iodine near pH 7 the only significant absorption seen besides that of the fully oxidized porphyrin (V) is at 500 mμ. If the extinction coefficient of Structure IV is about 10^4, then 3 mole per cent of Structure IV could have been detected.

To prove that this absorption at 500 mμ is due to Structures II and III, 2,2′,4,4′-tetramethyldipyrrylmethene (VIII, Fig. 4) was studied as a model. The acid form in aqueous solution has a band at 463 mμ, $\epsilon = 8.8 \times 10^4$, the



VI

FIG. 3



VII VIII

FIG. 4. Reaction 1

free base has a very broad band at 438 mμ, $\epsilon = 3.1 \times 10^4$, and its pK is at 8.4. The zinc complex has a sharp band at 480 mμ, $\epsilon = 10^5$ (per mole of dipyrrylmethene, in 50 per cent alcohol. The methene forms a colorless complex with sulfite ion. The equilibrium constant of Reaction 1 (Fig. 4) is about 3×10^{-6} mole liter^{-1} at 22°, and at an ionic strength of 0.2. An even tighter complex is formed with dithionite ion.

That the colorless compound formed is Structure VII and not the corresponding dipyrrylmethane produced by reduction with sulfite or dithionite ions is proved by the insolubility of the complex in ether and the immediate formation of Structure VIII on acidifying. Reduction to the colorless dipyrrylmethane occurs with potassium borohydride. This product is easily extracted with ether, is only slowly autoxidized to Structure VIII on acidifying, but is immediately oxidized by iodine. No complex is formed with Structure VIII and GSH or iodide ion at pH 7.

The properties of the intermediate in the oxidation of URO'gen which absorbs at 500 mμ are similar to those of the alkyl dipyrrylmethene with respect to the following: (1) spectral absorption, (2) pK value, (3) zinc complex, and (4) sulfite and dithionite complexes.

Spectral Absorption—The displacement of the band from 485 mμ for Structure VIII to 500 mμ for the intermediate is likely, due to the replacement of two alkyl groups by pyrrylmethyl groups, and to the enforced cis position of the rings. The extinction coefficient of the intermediate absorbing at 500 mμ may be estimated from the fact that the plot of the 560 or 610 mμ absorption of the porphyrin formed *versus* fraction oxidized with

TABLE II

Spectral Data

Compound		$m\mu_{max}$	ϵ	pK
URO'gen, I, Fig. 2		~202	$\sim 5 \times 10^4$	
UROporphomethene, II–III, Fig. 2	Acid	500	$\sim 10^5$	~8.8
	Free base	460	$\sim 10^4$	
	Zn complex	520		
2,4,2′,4′-Tetramethyldipyrrylmethene, VIII, Fig. 4	Acid	463	8.8×10^4	8.4
	Free base	438	3.1×10^4	
	Zn complex*	480	$\sim 1 \times 10^5$	
2,4,2′,4′-Tetramethyl-3,3′diethyldipyrrylmethene	Acid†	485	8.3×10^4	
	Free base†	442	2.1×10^4	
	Zn complex†	503	1.5×10^5	
Tripyrrylene, VI, Fig. 3	" "	627		
Mesobiliviolin	Acid	607	2.1×10^4	

All spectra obtained in water unless otherwise indicated.

* This spectrum in 50 per cent alcohol-water.

† These spectra in dioxane.

iodine is linear (Fig. 1) and the breaking point corresponds to a hexahydro reduction level. Therefore the molar amount of intermediate must be within the experimental error, *i.e.* ~5 per cent of the total porphyrin. If this amount of intermediate is present when the optical density at 500 mμ is a maximum, then $\epsilon = 2 \times 10^5$. Another estimate can be made by considering the initial slope of the 500 mμ absorption due to this intermediate. If this corresponds to a mixture of the tetra and dihydro stages (Structures II and III), *i.e.* to an average loss of 3 hydrogens, and if about 5 per cent of the iodine is used here, then $\epsilon = 1-2 \times 10^5$. Although these estimates may be in error by a factor of 2, they clearly show the expected order of magnitude. Table II summarizes the spectral data of these various compounds.

pK Value—The pK of the intermediate was measured by adding a so-

lution of the partially autoxidized URO'gen to buffer solutions of various pH levels. Above pH 9, a broad, less intense band at about 460 mμ replaces the 500 mμ band, again in agreement with Structures II and III. Due to autoxidation, the pK could only be estimated to be between 8.5 and 9. By titrating COPRO'gen with iodine at various pH levels between 6.8 and 9.6 and plotting the relative maximal 500 mμ absorption due to the intermediate *versus* pH, the pK of the intermediate is found to be 8.8 ± 0.2 (Fig. 5). Although the measured pK is probably some average of

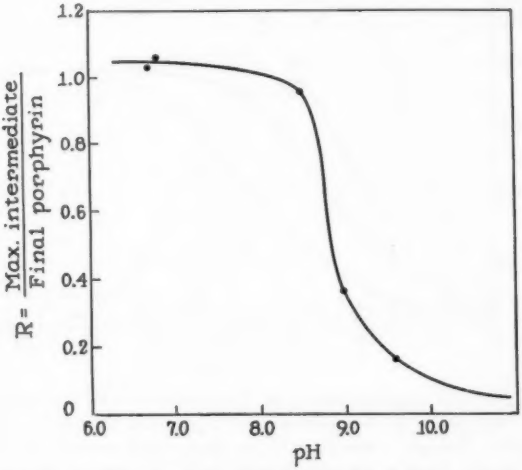


FIG. 5. Solutions of COPRO'gen were titrated with I_2 as described under Fig. 1, but at various pH levels. The maximal absorption observed at 500 mμ due to the intermediate (divided by the final COPRO concentration to normalize the various solutions) is plotted against pH.

the pK of Structure II and the two pK values of Structure III, it is sufficient to show the close similarity to the model Structure VIII.

Zinc Complex—The addition of zinc ion to the intermediate at slightly alkaline pH causes the band to shift from 500 to 520 mμ. This shift is the same as that found in the alkyl dipyrrylmethenes mentioned earlier.

Sulfite and Dithionite Complexes—The 500 mμ absorption of this intermediate is lost on addition of sulfite or dithionite ions; only the spectrum of the porphyrin remains. This 500 mμ absorption is not affected by GSH or iodide ion.

The above evidence supports the formulation of the intermediate oxidation products of the porphyrinogens as having the dipyrrylmethene Structures II and III. They shall hereafter be referred to as porphomethenes.

Autoxidation—The impure porphyrinogens are very sensitive to light and air, but become more stable on purification (21). In this respect they resemble most leuko dyes whose autoxidation appears to be photocatalytic. The autoxidation of URO'gen at pH 7 is in fact extremely sensitive to light (Fig. 6). During the autoxidation at pH <8.5 a transient absorption at 500 mμ having the same properties as that produced on oxidation with iodine is observed. The oxidation of the porphomethene first produced is autocatalytic (Fig. 6, Curve B′). That the fully oxidized porphyrin is

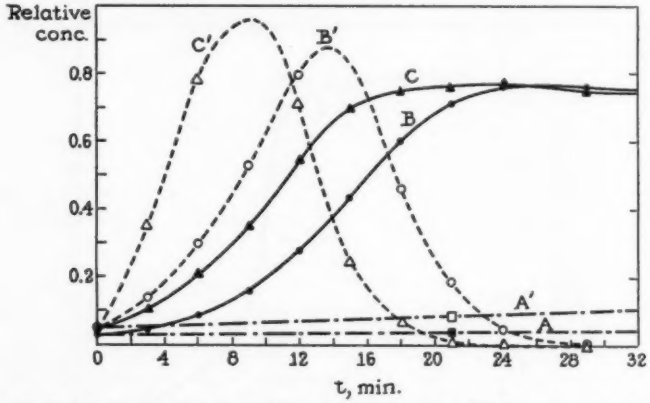


FIG. 6. Catalysis of photooxidation of URO'gen by URO. Samples A and B 1.1×10^{-4} M URO'gen in M/15 phosphate buffer, pH 6.8; Sample C, Sample A plus 5×10^{-6} M URO. Sample A was kept in the dark, while Samples B and C were illuminated at 10 cm. from a 100 watt Mazda bulb. Curves A, B, and C, relative porphyrin concentration of Samples A, B, and C. Curves A′, B′, C′, relative porphomethene concentration of Samples A, B, and C. The porphomethene concentration has been magnified about three times relative to the porphyrin concentration. The constant increment due to the added URO has been subtracted from Curve C. See under "Experimental."

at least one of the photoactive species is also shown in Fig. 6. The addition of 5 mole per cent of URO to the URO'gen solution causes a marked shortening of the induction periods (Fig. 6, Curves B′ and C′). Attempts to determine an action spectrum by using the Cary or Beckman spectrophotometers were unsuccessful. No direct proof that the porphomethene is photoactive was obtained. However, photoactivity might be expected, since solutions of dipyrrylmethenes are very photolabile.

If after partial photooxidation the solution is returned to the dark, the oxidation rate decreases to a value approximately that of a control kept in the dark.

Solutions of URO'gen from which O_2 has been thoroughly removed by

freeze-thawing *in vacuo* are only slightly oxidized on exposure to intense light.

The photooxidation is inhibited by 10^{-3} M GSH, 2-mercaptoethylamine and sodium sulfite at pH 7. An example of the inhibition by GSH is given in Fig. 7. The inhibition by sulfite ion is complicated by concurrent destruction of the porphyrin, resulting in yields of less than 50 per cent. The

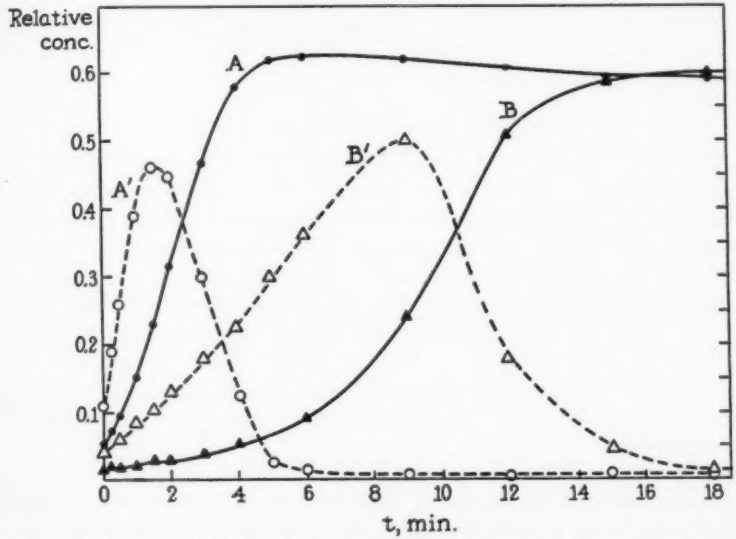


FIG. 7. Inhibition of photooxidation with GSH. Sample A contained URO'gen at 8.7×10^{-6} M in M/15 phosphate buffer, pH 6.8. Sample B was A plus 5×10^{-3} M GSH. Both were illuminated at 20 cm. from a No. 2 photoflood lamp. Similar samples kept in the dark showed less than 5 per cent oxidation during this period. Curves A and B, relative porphyrin concentration of Samples A and B; Curves A' and B', relative porphomethene concentration of Samples A and B. The porphomethene concentration has been magnified about 2.5 times relative to the porphyrin concentration. See under "Experimental."

photooxidation is catalyzed by ferric ion in 1 M HCl but not in phosphate buffer at pH 6.8. EDTA has no effect at pH 6.8. Nitrobenzene, *p*-phenylenediamine, picric acid, and potassium iodide do not inhibit at 10^{-5} M. The photooxidation rate increases with decreasing pH, and the yield of porphyrin also increases from about 50 per cent in 0.1 M NaOH, to 75 per cent near neutrality, and to over 90 per cent in 1 M HCl.

Since the porphyrins can also be photoreduced in the presence of various reductants such as GSH, the mechanism of these photoreactions requires further study.

Under conditions whereby the yield of porphyrin on photooxidation of the porphyrinogen is not quantitative, a 635 mμ band arises on photooxidation. At pH 7 it attains an intensity of about one-quarter of that of the final 560 mμ porphyrin band, and is quite irreversible. It is not affected by excess iodine. It is displaced from 635 mμ at pH 7 to 644 mμ in alkali and to 618 mμ in acid. It is therefore not a dihydroporphyrin of the tripyrrylene type (Structure IV). From the autoxidation of URO'gen from turacin feathers, Nicholas and Rimington isolated a similar pigment and called it a chlorin (24). However, these properties are also in accord with those of an oxyporphyrin (25).

Enzymic Decarboxylation of URO'gen

Product—Evidence that the final product of the action of this enzyme on URO'gen is a porphyrinogen is given below. This porphyrinogen was shown to be COPRO'gen by quantitative oxidation with iodine and identification of the resulting porphyrin as COPRO by (1) its HCl number; (2) the characteristic shift of the Soret band in acid and the relative intensities of the visible bands near neutral pH on going from URO to COPRO (Table I); (3) paper chromatography of both the free COPRO and its tetramethyl ester.

Specificity—The enzyme preparation does not decarboxylate porphobilinogen, δ-aminolevulinic acid or uroporphyrin (see under "Experimental" and Table III).

Porphyrins with 7- to 5-Carboxyl Groups—The porphyrins with 8- 2-carboxyl groups may be separated by paper chromatography with lutidine (16). On partial enzymic decarboxylation of URO'gen and oxidation of the products, at least three spots corresponding to the porphyrins with 7, 6-, and 5-carboxyl groups appear (Fig. 8). The R_F values do not seem to be a linear function of the number of carboxyl groups. The porphyrins with 7- and 6-carboxyl groups remain at a low and roughly steady state concentration for a long time while porphyrin with 5-carboxyl groups is present in relatively low concentration. At a very low initial substrate concentration, the partially decarboxylated products are finally completely converted to COPRO. These intermediates amount to about 5 per cent of the isolated COPRO for isomer III and about twice that amount for isomer I. Since the rate of COPRO'gen formation from the various isomers of URO'gen varies only by a factor of 2 (see below), this preparation probably decarboxylates the acetic acid side chains at random. Thus the porphyrinogen having seven carboxyl groups obtained from URO'gen isomer III could be a mixture of four isomeric compounds, every one of which would, on continued decarboxylation, finally yield COPRO'gen isomer III. Considering all four isomers of URO'gen, there would be a

total of thirty-four different substrates for this enzyme: five, five, fifteen, and nine in the I, II, III, and IV series, respectively. The number of en-

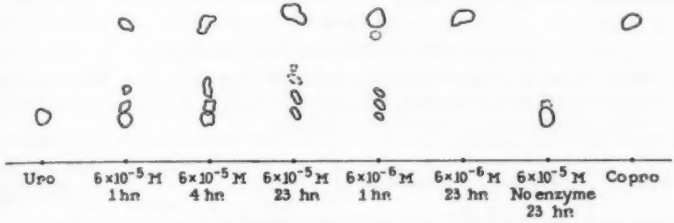


FIG. 8. URO'gen and the enzyme were incubated (see under "Experimental") at the indicated concentrations and for the indicated times. After oxidation with iodine, the unfractionated porphyrins were paper-chromatographed with 10 parts of 2,6-lutidine:7 parts water by volume as solvent. The jar also contained a beaker with 6 M NH_3.

TABLE III

Enzyme, URO'gen, and URO

Activity of enzyme with URO and URO'gen I and III. URO'gen or URO and a concentrated enzyme preparation or buffer were incubated as described under "Experimental," but under N_2 in Beckman or Cary cells with greased glass stoppers. The spectra were rapidly measured at the end of the incubation period; 16 hours for all but the last experiment, which was 4 hours and in which a different enzyme preparation was used. The cells were then opened and the solutions titrated with iodine. The solutions were then processed as described in the text.

Substrate	Amount at end of incubation		Amount isolated after iodine titration	
	Porphomethene	Porphyrin	URO	COPRO
	mμmoles	*mμmoles*	*mμmoles*	*mμmoles*
URO'gen I	340	15	55	130	52
" I + 10^{-3} M $Na_2S_2O_4$	340		2	110	46
" III	400	20	90	38	210
" III + 10^{-3} M $Na_2S_2O_4$	400		3	41	192
URO III	400			316	0.6
" III + 10^{-3} M $Na_2S_2O_4$	400			274	0.7
URO'gen III, no enzyme	400			287	0.7
" III + 5×10^{-3} M GSH	540	8	25	177	168

zymes involved in these decarboxylations is unknown. For convenience, however, we shall refer to the preparation as "the enzyme," uroporphyrinogen decarboxylase, or UD-ase.

Isomers—The data of Tables III and IV show that the yield of COPRO'gen from the various isomers of URO'gen is III > IV > II > I. Isomer III

is decarboxylated twice as fast as isomer I (Fig. 9). Analysis of both the recovered URO and of the COPRO showed the expected isomers, although

TABLE IV

Enzymic Decarbozylation of URO'gen Isomers

A mixture of the URO'gen with GSH at 5×10^{-3} M and 5 ml. of the enzyme solution was incubated under nitrogen for 23 hours at 38°. Solutions were processed and the isomers identified as described in the text.

Isomer	URO'gen	Concentration	Amount recovered		Per cent COPRO of recovered URO + COPRO
			URO	COPRO	
	mμmoles	*M* × 10^5	*mμmoles*	*mμmoles*	
I	440	5.0	190	41	18
II	220	2.6	90	29	24
III	270	3.1	69	71	51
IV	380	4.3	110	53	32

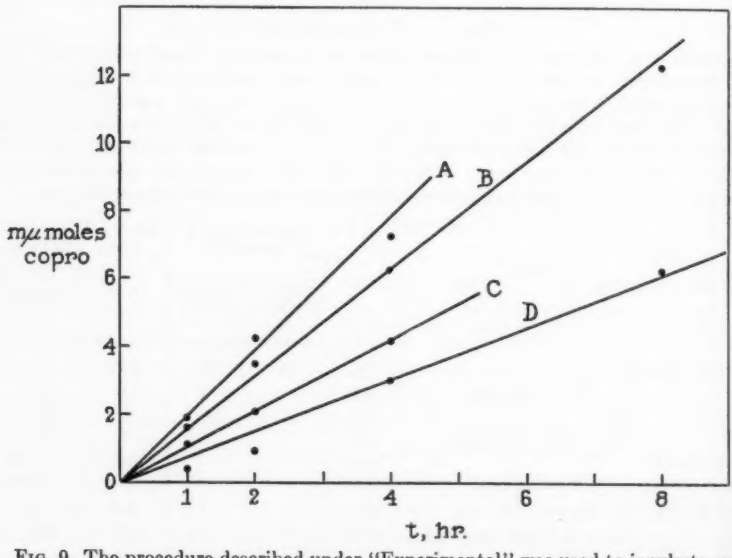


FIG. 9. The procedure described under "Experimental" was used to incubate and to assay for the resulting COPRO. Curve A, 1.9×10^{-5} M URO'gen III; Curve B, 5.7×10^{-5} M URO'gen III; Curve C, 1.8×10^{-5} M URO'gen I; Curve D, 5.4×10^{-5} M URO'gen I.

I and II (similarly III and IV) cannot be distinguished by these methods. Incubation of equimolar mixtures of URO'gen I and III with the enzyme preparation gave mixtures of COPRO I and III at a rate intermediate be-

tween that of I and III alone. The III isomer always predominated in the product.

Chemical or enzymic isomerization, by ring opening and reclosure in differing positions, would lead to a randomization of the sequences of the pyrrole units, thus changing the isomer type. An example of this type of isomerization occurs on reducing mesoporphyrin with zinc in acetic acid (26). The completely randomized mixture of URO'gen or URO would contain one-half isomer III, one-fourth isomer IV, one-eighth isomer II, and one-eighth isomer I (6). The observation of no change in the recovered URO isomers I and II and the isolation of the corresponding COPRO isomers I and II show that little, if any, of this isomerization took place on reduction with sodium amalgam, incubation with enzyme, and oxidation with iodine. The present methods of isomer analysis are not quantitative, but it can be estimated that less than 10 per cent isomerization of I to III took place.

TABLE V

Activity of Enzyme with Various Substances

Substance	Concentration	Relative activity	
		Alone	5×10^{-3} M GSH
	$M \times 10^3$		
Mg^{++}	0.2	0.95	
"	1.2		0.99
Ca^{++}	1.2		1.03
Zn^{++}	0.12	0.75	0.73
α,α'-Dipyridyl	1.2	0.99	
8-Hydroxyquinoline-5-SO_3H	1.2	0.90	
EDTA	1.2	0.68	0.77
SO_3^{--}	1.2	1.29	1.31
$S_2O_4^{--}$	1	1.0	
HCO_3^-	12		0.91
Porphobilinogen	1.2		0.8
Indoleacetic acid	5		1.0
COPRO	0.0064		0.8
COPRO'gen	0.0064		0.9
O_2		0.6	
URO	0.054	0.5	0.9

Incubation and assay were as outlined in the text.

Activity—The activity of the enzyme is largely unaffected by Ca^{++}, Mg^{++}, Zn^{++}, α,α'-dipyridyl, 8-hydroxyquinoline-5-sulfonic acid, EDTA, SO_3^{--}, $S_2O_4^{--}$, HCO_3^-, porphobilinogen, indoleacetic acid, and low concentrations of COPRO or COPRO'gen (Table V). Oxygen inhibits due to autoxidation of the URO'gen. The inhibition by URO is prevented by

GSH and hence is very likely due to sensitized autoxidation of the URO'gen by URO. Strong inhibition occurs with Hg^{++}, Cu^{++}, Mn^{++}, iodoacetamide, and *p*-chloromercuribenzoic acid, but is in all cases prevented by excess GSH (Table VI). The preparation is thus a typical enzyme with essential sulfhydryl groups, which either does not require a metal, or, if so, binds it very tightly.

The activity of the enzyme preparations used in most of the experiments was not greatly increased (about 20 per cent) by added GSH. It was found, however, that when two streaks were run on the starch plate the fractions closest to the anode were five times more active in the presence

TABLE VI

Effect of Inhibitors on Enzyme Activity

Inhibitor	Concentration	Relative activity	
		Alone	5×10^{-3} M GSH
	M × 10^4		
Cu^{++}	0.012	1.0	
"	0.12	0.1	
"	1.2	0.01	0.25
Hg^{++}	0.012	0.93	
"	0.12	0.01	
"	1.2		0.96
Mn^{++}	1.2	0.12	1.1
Iodoacetamide	12	0.05	1.0
p-Chloromercuribenzoic acid	7	0.02	1.0

Incubation and assay were as outlined in the text.

of 5×10^{-3} M GSH. The half saturation level of GSH was about 10^{-3} M. When the electrophoresis was run on starch prewashed with bovine albumin (12), this effect was not observed. Exposure of the preparation to metal contamination increased the dependence of the activity on GSH, *e.g.* dialysis in Visking tubing gave a completely inactive product, which was partly reactivated by GSH. To protect against both trace heavy metals and autoxidation, GSH was usually added to the incubation mixture.

Incubation of the enzyme with 6×10^{-6} M COPRO'gen and 5×10^{-3} M GSH in 0.01 M bicarbonate buffer at pH 6.8, 38°, for 4 hours under N_2 gave no trace of porphyrins with more (or less) than four carboxyl groups, showing the expected irreversibility of the reaction.

Optimal pH—The optimal pH for the over-all reaction is about 6.8, and is not affected by changing from aerobic to anaerobic conditions, although

the yield of COPRO is increased 2-fold under the latter conditions (Fig. 10). Changing from phosphate to Tris or bicarbonate buffer near the optimal pH had no effect.

Substrate Concentration—The rate of COPRO'gen formation appears to decrease at a URO'gen concentration greater than about 2×10^{-5} M (Fig. 9). Experiments with lower substrate concentrations were difficult to carry out due to the low activity of the preparation, and because of the

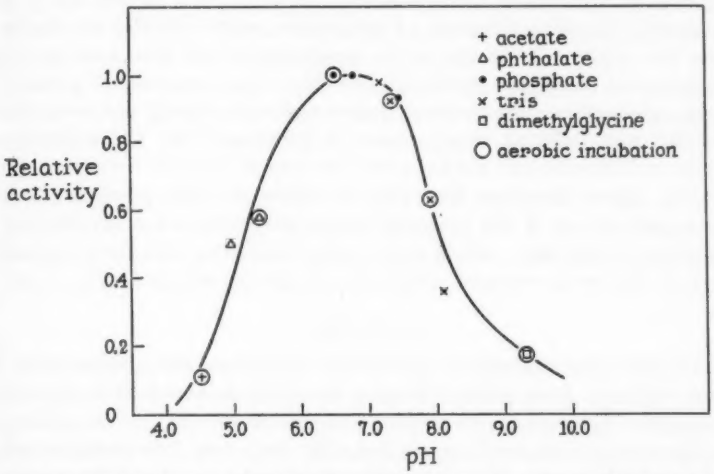


FIG. 10. 1 ml. of enzyme solution and 0.2 ml. of 0.1 M buffer were adjusted to the proper pH, and 0.2 ml. of URO'gen at the same pH was added. After incubation, the pH was redetermined and, if no significant change had occurred, the solution was processed as described under "Experimental." Circled points, solution incubated aerobically for 3 hours at 38°, normalized to pH 6.5. Other points, solution plus 5×10^{-3} M GSH incubated for 4 hours anaerobically at 38°, normalized to pH 6.8. Two different enzyme preparations were used.

limitations of the method of analysis. They do indicate that the apparent Michaelis constant is less than 5×10^{-6} M. Since intermediates do not accumulate (see above), the first decarboxylation of URO'gen is essentially the rate-determining step. Neve, Labbe, and Aldrich, on adding URO'gen to lysed duck cells and measuring Fe^{59} incorporation into heme, also found maximal activity at a URO'gen concentration of about 10^{-5} M (7). This may be due to substrate inhibition or to inhibition by partially oxidized porphyrins. It is not due to product inhibition, since the rates were linear for over 8 hours (Fig. 9) and added COPRO'gen at similar concentrations did not inhibit the reaction (Table V).

Substrate—The substrate for this enzyme is clearly a reduced porphyrin

(Table III). URO is decarboxylated at less than 1/100th the rate of the URO'gen. Since some of the porphomethenes were usually present at the end of an incubation with the enzyme, the question of their activity as substrates arises. That they are not obligatory intermediates is shown by the following. (1) The direct isolation of COPRO'gen in over 90 per cent yield by ether extraction of short time incubations as described under "Experimental." Four experiments showed that 25 ± 8 per cent of this material was totally oxidized COPRO, the remainder having 5.5 ± 0.6 hydrogens by iodine titration. Furthermore, more COPRO was isolated from the mixture at the end of the incubation period than total porphomethene and porphyrin present (Table III). The quantities of porphyrin and porphomethene were estimated spectrophotometrically and would have included any URO at these stages of oxidation. (2) The addition of sulfite or dithionite ions did not affect the rate of the decarboxylation (Table IV). Since these ions form tight complexes with the porphomethenes, the concentration of the porphomethenes is therefore not involved in a rate-determining step. All of this evidence indicates that the actual substrate is the totally reduced porphyrin, *i.e.* the porphyrinogen.

DISCUSSION

The somewhat contradictory evidence concerning the intermediates in heme synthesis from porphobilinogen has been summarized in the Ciba Foundation symposium on porphyrin biosynthesis (27). Practically all the observations are easily reconciled with the actual intermediates being the porphyrinogens. The porphyrins produced on autoxidation are inactive in the enzyme systems and so accumulate. The amount accumulated will depend not only on the activity of the enzymes in the sequence but also on the amount of light present and on the presence of catalysts or inhibitors of the autoxidation. The autoxidation *in vivo* of these porphyrinogens is probably not important in the bone marrow since this makes a reasonable dark room. In other cells and in plants, the autoxidation may be inhibited by antioxidants, *e.g.* GSH. If the enzymes involved have low Michaelis constants and high turnover numbers, the concentration of free porphyrinogens at any time will be very low. The apparent Michaelis constant for the rabbit URO'gen decarboxylase is $<5 \times 10^{-6}$ M.

Since the enzyme decarboxylates URO'gen I to form COPRO'gen I, the occurrence of COPRO I in chronic porphyria is readily explainable, assuming that the enzyme from human cells has the same properties. A defect must occur in the porphobilinogen condensation to give some of the I isomer. The amount of COPRO I formed will depend, *inter alia*, on the rate of autoxidation of URO'gen I.

Since URO'gen is the actual substrate, we now have indirect evidence

that the product of the condensation of porphobilinogen is also URO'gen. Similarly, COPRO'gen would lead to protoporphyrin, and in fact we have directly confirmed this with preparations from red cells and *Euglena* (28).

The porphyrinogens cannot bind metals since they are simply four pyrrole rings joined by four methane bridges. Therefore incorporation of metal into heme or chlorophyll must take place during or after the oxidation to porphyrins. In aqueous solution at pH 7, ferrous ion readily coordinates with monomolecularly dispersed porphyrins; but magnesium ion does not and a special mechanism may be required.

If the byproduct of the photooxidation of porphyrinogens absorbing at 635 mμ could be shown to be a chlorin rather than an oxyporphyrin, this reaction might represent a step in the synthesis of chlorophyll.

We wish to acknowledge the able technical assistance of Mrs. Annabelle Long and Mr. William Cumming. We thank Dr. S. F. MacDonald and Dr. C. Rimington for the gift of some porphyrins, and Dr. A. Corwin for the sample of dipyrrylmethene.

SUMMARY

An enzyme preparation which converts uroporphyrinogen to coproporphyrinogen has been isolated from rabbit reticulocytes by zone electrophoresis on starch. Uroporphyrinogen I is decarboxylated at about one-half the rate of isomer III. Porphobilinogen, δ-aminolevulinic acid, and uroporphyrin are not decarboxylated by this preparation.

The autoxidation of uroporphyrinogen is photocatalytic and is sensitized by the product, uroporphyrin. An intermediate stage in the oxidation of uroporphyrinogen characterized by intense absorption at 500 mμ is shown to have a dipyrrylmethene type structure. Evidence is presented that this "porphomethene" is not involved in the enzymic decarboxylation.

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-3 PUSH-BUTTON SUPERSPEED CENTRIFUGE. Fully automatic, fully en-sed. SERVALL-BLUM "Gyro-Action" Self Centering Drive sets new ndards of performance. Smoothly and automatically accelerates to de-ed preset speed — accurately times the operation for you — then dynamlly brakes itself at the completion of the run. Even stops itself safely d automatically in the event of excessive imbalance in loading. Easily : safest, most convenient and versatile instrument available today. Solves problem of separating small amounts of precipitate from large volumes solution with the SERVALL "Szent-Gyorgyi & Blum" Continuous Flow stem.* Sealed batch operations with 400 ml. SS-34 angle rotor (over ,000 rpm — 34,800 x G), and 2,200 ml. GSA large-capacity rotor (9,500 n — 14,600 x G). Further versatile rotors nearing completion. Armor te enclosed rotor compartment. Low silhouette. Built-in ammeter-tachoter, speed setting control, synchronous motor driven timer, SERVALL oisuppressor". Facility for remote control.

ite for bulletin BC-6S3

SS-4 ENCLOSED SUPERSPEED CENTRIFU For those who prefer a manually controlled model. The same advanced features, smooth performance and rotor versatility as the SS-3, including SERVALL "Szent-Gyorgyi & Blum" Continuous Flow System. Entire panel slides out for convenient remote control and includes built-in ammeter-tachometer, synchronous motor driven timer and variable transformer. Armor plate guard, SERVALL "Noisuppressor", low silhouette, rotor imbalance cut-off.

e Canadian distributor: Cave and Company, Ltd., Vancouver, Edmonton and Toronto

* Patents pending

Ivan Sorvall, Inc. Norwalk, Connecticut, U.S.A.

Since 1930 designers, manufacturers, distributors of SERVALL laboratory instruments

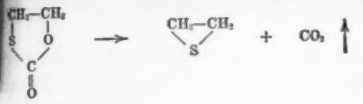
...AMONG SOME 3600 ORGANICS

, ethylene sulfide

bout your polymer success stories. Polyethylene- (—CH_2CH_2S—)$_n$ is one that never made good. r want of trying. Been subject of much litera- Few suitable solvents. Monomer, ethylene sul- (CH_2—CH_2) is clear, volatile liquid (BP 56).

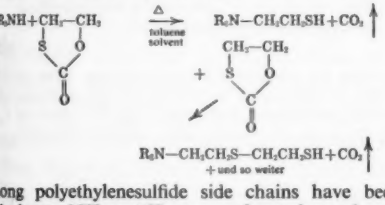
't stay that way long. Maybe a day if you play cards right. Used to be prepared by reacting roethylmercaptan with sodium bicarbonate. ne sulfide has to be fractionated from the water. of it polymerizes on the way. Russians do it by g ethylene oxide with potassium thiocyanate. drawn out and messy. Tried it here and got no- near the 25-50% yields they claim. May be ult.

yway, spent long time looking for better idea. d up inventing "new composition of matter." it *Ethylene Monothiocarbonate*. Nice, smelly but liquid. Keeps long time below 200 C. Get it hot and presto, ethylene sulfide. So:



% yield.

thylene sulfide long considered *sine qua non* of rcaptoethylation. Now you can mercaptoethylate . Life thus made simpler, more satisfying, safer use at temperature required for attachment of CH_2CH_2SH group, ethylene sulfide is far above ling point but *Ethylene Monothiocarbonate* is not. action proceeds peaceably without pressure plumb- and all that jazz. Mercaptoethylation might be hed onward and onward. Viz.,



Long polyethylenesulfide side chains have been ooked onto NH_2 or SH groups of proteins and onto e OH groups of cellulose. Also talk in literature of twining natural polymer chains with polyethylene-sulfide chains formed *in situ* but not bonded to them.

Glad to sell you *Ethylene Monothiocarbonate*. From $5.15 for 10 grams on up in quantity and down in price. Ask for Eastman 7367.

Unfetid imagination

Across the continent year in and year out rumble the trucks and boxcars bringing to our parent silver ingots for his photographic emulsions. Nobody is as interested in silver as he. At this year's Pittsburgh Conference on Analytical Chemistry, three of his chemists told how rapidly, simply, and well-nigh automatically they titrated it using a silver sulfide-calomel electrode pair with a solution of *Thioacetamide* (Eastman 1719), the unfetid, up-to-date source of sulfide ion for the analyst. They have worked the method with mercury as well. The mercury is complexed by ethylenediaminetetraacetate, the silver by thiosulfate. If some other heavy metal meant as much to you as silver and mercury do to these chaps, you would ask us for an abstract of their presentation and then try to imagine the proper indicator electrodes for *your* metal.

Glucosamine

Because of an item we read in the *Wall Street Journal* recently, a copy or abstract of which we refuse to supply, we predict that this year will see an upsurge of interest in the biochemistry of glucosamine.

This amine sugar is easy to prepare. The classical starting material is chitin, and the essence of the method is acid hydrolysis. Chitin can be obtained in several ways. Perhaps the most efficient of these is to go around to the back door of the most prosperous sea food restaurant within easy reach and talk to the official in charge of the garbage cans about a deal for his vacant lobster shells. A slower method is to put shrimp on the family menu as often as necessary and save the peelings. Investigators with a distaste for sea food can get their chitin at this season of the year by putting a corps of small boys to work hunting June bugs (May bugs, that is, in the South).

For those who shun behavior tending to mark them as colorful scientific personalities, there is still another alternative. They can purchase ready-purified D-*Glucosamine Hydrochloride* as Eastman 644. Foreseeing the demand from the shy ones, we have laid in a stock of clean chitin that looks like breakfast food or perhaps fingernail clippings.

rice is subject to change without notice.

Each one of the 3600 or so Eastman Organic Chemicals available fror *Distillation Products Industries*, Rochester 3, N. Y., has its own story

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* Ref. U. S. Armed Forces Medical Journal, Vol. 5, Pg. 693, May, 1954.

This accurately prepared powder concentrate, when dissolved in distilled water, will make fresh Drabkin solution (in accordance with U. S. Armed Forces specifications) for the determination of hemoglobin.

The exact amount of powder for making one liter of Drabkin solution is packaged in individual glass vials. A box of 12 vials, sufficient for preparing 12 liters of solution, occupies a space of only 6¾" long, 2½" wide and ¾" high.

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LABORATORY APPARATUS
REAGENTS AND CHEMICALS

An Editorial

A forward step

Advances in the diagnosis and treatment of diseases have increased the importance of biochemical determinations. Yet, biochemical techniques have not always advanced at the same rate.

To facilitate such advances in diagnosis, increased accuracy is necessary. This is being approached in two ways.

First, the degree of accuracy of usual methods is being questioned. Professional associations in the laboratory field are currently re-evaluating methods and recommending those which are superior.

Second, increased attention is being given to the importance of routinely using reference standards, whatever method is used. Reference standards are required to calibrate instruments and prepare curves. They are needed to control the method used.

Now, a group of prominent laboratories has questioned the reliability of usual reference standards themselves. These investigators feel that, to be trusted, the reference standard must closely duplicate the unknown. It must be reliable without question. It must be adequately stable.

Their field testing of the reference standard, Versatol, under routine conditions, proved this material ideal. To the credit of the six participating laboratories is their admission of repeated variance in results from the values given for Versatol. Recheck in every case proved malfunctioning instruments or reagents, rather than Versatol in error. Such open-minded routine use of Versatol in all laboratories can help in obtaining higher levels of accuracy in biochemical determinations.

Chemists report new means to avoid errors

Six Leading Clinical Chemists Collaborate In Evaluation

New York, June, 1958—T June issue of the journal, Cl ical Chemistry, carries the fi report of an extensive stu evaluating reference standa for use in biochemical det minations.

Directors of the clinical che istry laboratories of six N York hospitals participated a collaborative study to det mine the requirements for ideal clinical chemistry cont Warner-Chilcott's Versatol v evaluated against these quirements.

Versatol was subjected to ri tests for reliability, reprod ibility and stability. It was a checked for chemical and ph ical similarity to serum. T results were statistically ev uated.

The study showed that Versa meets all of the requireme of the ideal reference stand for biochemical procedures is far superior to all materi previously evaluated.

Versatol

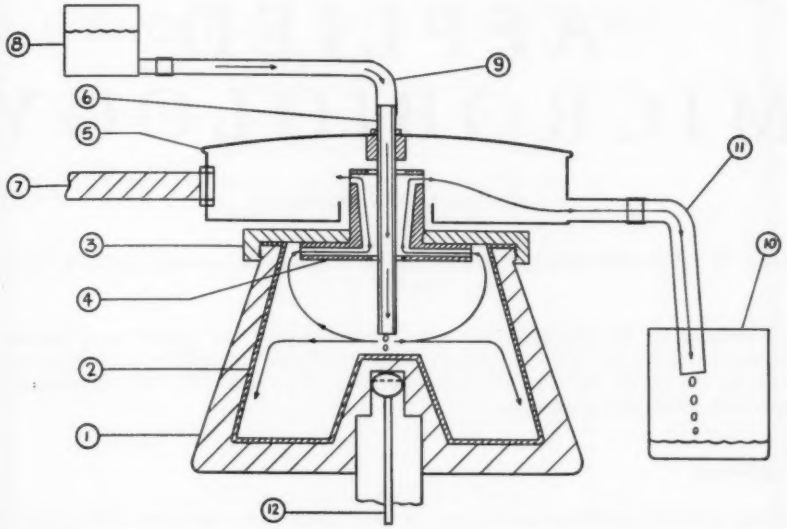
TRADEMARK

LABORATORY SUPPLY DIVISION • **WARNER-CHILCOTT,** MORRIS PLAINS, N

*CONTINUOUS FLOW**

Super-Speed CENTRIFUGE ROTORS

(Pat. app'd for)



Part Nos. 1, 2, 3, 4 and 12 rotate. All others are stationary

***GREATER CAPACITY** **SIMPLIFIED OPERATION** **FASTER FLOW RATE** **LOWER COST** **REMOVABLE POLYETHYLENE ROTOR LINER**

To commence operation merely insert polyethylene liner into rotor and lower collector with attachments.

Materials to be centrifuged flow from reservoir 8 into sealed rotor 1 through central inlet tube 6. Heavier particles will impinge upon the angular wall and travel downward. Lighter mediums are continually ejected through outlet ports in the discharge cap 4 into the collector 5 from whence they flow to the collection chamber 10 (see arrows).

Removable rotor liner permits quick replacement for continued operation without the need for messy rotor cleaning. Due to design simplicity, complete assembly and disassembly time is reduced to a minimum. Materials centrifuged come in contact with only the polyethylene liner, stainless parts and inert tubing.

Continuous Flow Rotors AVAILABLE IN TWO SIZES.

ROTOR CAT. NO.	CAPACITY (approx.)	MAX. SPEED (approx.)	MAX. FORCE (approx.)
CFR-1	800cc	17,000 RPM	35,000 × Gravity
CFR-2	2000cc	11,000 RPM	20,000 × Gravity

OTHER NEW PRODUCTS

- **FULLY AUTOMATIC SUPERSPEED CENTRIFUGES (two models)**—Accommodate all rotors including new continuous flow. Both models include controls for automatic rotor acceleration, push button dynamic braking, rotor unbalance trip and many other features.
- **THREE LITER ROTOR**—First rotor ever to spin such capacity at forces in excess of 10,000 × Gravity.
- **VOLUMIXER**—Sealed, powerful, high speed homogenizing within 1 Gal. or 2 Gal. containers.

WRITE FOR ADDITIONAL DETAILS. REFER TO JBC-78

PARTS LIST

1. Rotor
2. Polyethylene liner
3. Cover (stainless steel)
4. Discharge cap (SS)
5. Collector (S.S.)
6. Inlet Tube (S.S.)
7. Collector support arm
8. Reservoir
9. Plastic tubing (inlet)
10. Collection chamber
11. Plastic tubing (outlet)
12. Motor drive

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CONTENTS—Continued

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